

THE PHARMACOLOGY OF THREE INWARDLY RECTIFYING POTASSIUM
CHANNELS IN NEONATAL RAT CARDIAC MYOCYTES.

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ABSTRACT

The aim of the present study was to investigate the pharmacology of three inwardly rectifying K^+ -channels in neonatal rat cardiac myocytes, $I_{K_{ACh}}$, I_{K_1} , $I_{K_{ATP}}$ using whole cell voltage clamp techniques. Cells were held at -50mV.

A previous study has shown that clotrimazole, an antimycotic agent, and cetiedil, an antisickling agent are potent against the $I_{K_{ACh}}$ in atrial myocytes. Structural analogues of these compounds were tested on the three inward rectifiers.

UCL1880, an analogue of clotrimazole was found to be potent on the G-protein gated channel, $I_{K_{ACh}}$, with an IC_{50} of $0.18\mu M$. Concentrations which caused an almost maximum block of this current were relatively ineffective on the I_{K_1} and $I_{K_{ATP}}$. UCL1495, an analogue of cetiedil, had an IC_{50} of $0.46\mu M$ against the G-protein gated current. It was ineffective on the I_{K_1} and the $I_{K_{ATP}}$ current. Both of the compounds exhibited use-dependence but not voltage dependence.

Glibenclamide, a sulphonylurea, was tested on $I_{K_{ATP}}$ and $I_{K_{ACh}}$ as it has been suggested that the adenosine activation of $I_{K_{ACh}}$ includes the activation of $I_{K_{ATP}}$.

Glibenclamide was found to be far more effective on the $I_{K_{ATP}}$. The concentrations which were effective on $I_{K_{ATP}}$ did not block the $I_{K_{ACh}}$ or the I_{K_1} .

Barium was also tested on these three channels to see if there was any selectivity in its action. It had IC_{50} values of $34.9\mu M$ on $I_{K_{ACh}}$, $16.8\mu M$ on I_{K_1} and $164.5\mu M$ on $I_{K_{ATP}}$. The block on $I_{K_{ACh}}$ was voltage dependent, increasing at negative potentials, with an IC_{50} of $3.8\mu M$ at -120mV.

UCL1880 and UCL1495 are selective for $I_{K_{ACh}}$ and may be used as pharmacological tools to compare and contrast other G-protein gated currents to the native atrial channel.

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This work is dedicated to the memory of my father. Also to the memory of a dear departed friend. Thank you both for making such a difference in our lives. May God bless you.

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CHAPTER ONE : INTRODUCTION

CHAPTER ONE: INTRODUCTION

The surface membranes of cells contain proteins that facilitate the movement - be it exit or entry - of charged particles, ions, between the cell and its environment. Among the most important of these proteins are ion channels. In 1902, Bernstein suggested a selective potassium permeability in excitable cells and so can be credited with discovering potassium channels, at least in a general sense. Subsequently the majority of cells were shown to have some permeability to Na^+ , Ca^{2+} and Cl^- as well as to K^+ .

K^+ channels stabilize the membrane potential by maintaining it closer to the reversal potential for K^+ and away from the firing threshold of electrically excitable cells. Thus they set the resting membrane potential and tend to inhibit the initiation of action potentials - their opening reduces the effectiveness of excitatory inputs on a cell. K^+ channels can also play a part in the 'spacing' of trains of action potentials and this helps some excitable cells to generate rhythmic activity. These are not the only roles of K^+ channels as they are also involved in net transport for example in epithelia where they play a part in the movement of salt and water in and out of body compartments.

The main types of K^+ channels, their classification and their roles in various tissues are discussed in the following section. Most emphasis has been placed on the types of K^+ channels that are present in cardiac tissue.

1.1: VOLTAGE-ACTIVATED K^+ CHANNELS

1.1.1: DELAYED RECTIFIERS (I_K)

Hodgkin and Huxley (1952) discovered the existence of the delayed rectifier in squid giant axons. The delayed rectifier K^+ channels open in response to depolarisation after a delay and contribute to the repolarization phase of the action potential in many excitable cells. The current is activated at potentials positive to approximately -50mV and activation is complete between 0 and +20mV. In cardiac muscle the current flows mainly during the late plateau phase of the action potential to initiate rapid and final repolarisation of

the action potential. This channel is widely distributed in excitable and non-excitable cells.

Peptide toxins have been used extensively in the classification of K^+ channels. Dendrotoxin (*Dendroaspis angusticeps*) from the venom of African mambas blocks I_K in rat nodose ganglia (Stansfeld *et al.*, 1986) and dorsal root ganglion neurons of the guinea-pig (Penner *et al.*, 1986). The delayed rectifier in the frog node of Ranvier can be blocked by TEA applied either intracellularly with an IC_{50} of ~ 20 mM or extracellularly with an IC_{50} of ~ 0.4 mM (Armstrong and Hille 1972). The IC_{50} for TEA in *Myxicola* giant axons is 24 mM whereas even 250 mM externally applied TEA has no effect in squid giant axon (Armstrong, 1971).

In guinea-pig atrial cells there are thought to be two types of delayed rectifier channel with single channel conductances of 10 and 3 pS in symmetrical K^+ (Horie *et al.*, 1989). Several other studies have demonstrated the presence of two components - fast and slow- of this current for example in sheep cardiac Purkinje fibres (Noble and Tsien, 1969), bullfrog atrial myocardium (Goto *et al.*, 1985), and rabbit sino-atrial node (Noma and Irisawa 1976, DiFrancesco *et al.*, 1979). In guinea-pig ventricular myocytes the rapid component can be blocked by a class III antiarrhythmic (E4031, 5 μ M) and the slow component by the anaesthetics propofol (100 μ M) and thiopentone (100 μ M). The contributions of these two components to the total current were comparable (Heath and Terrar, 1996a). Two components of the delayed rectifier current have also been described in frog short skeletal muscle fibres (Lynch, 1985) and in frog oocytes expressing the human delayed rectifier K^+ channel (h-DRK1) (Bendorff *et al.*, 1994). The human K^+ channel gene, HERG, which has been linked to long-QT syndrome, is thought to encode the fast component of the delayed rectifier current found in the heart (Wymore *et al.*, 1997). A channel protein known as minK associates with another channel protein called KvLQT1 to produce a current which resembles the slow cardiac repolarisation current (I_{KS}) (Sanguinetti *et al.*, 1996). MinK forms a stable complex with HERG and regulate the rapidly activating cardiac delayed rectifier activity (McDonald *et al.*, 1997).

1.1.2: THE TRANSIENT (EARLY) OUTWARD CURRENT (I_A).

This current is referred to both as the transient (early) outward current and as the rapidly inactivating current. It was first described by Connor and Stevens (1971) in molluscan neurons. By holding the membrane potential at -40mV and then stepping to -5mV, the delayed rectifier and Ca^{2+} -activated currents were observed. By holding at -80mV and stepping to -5mV I_A was also activated - subtraction of these two currents gave the rapidly activating and inactivating I_A (Connor and Stevens, 1971). The current is active at the subthreshold stage of the membrane potential - during depolarisation after a period of hyperpolarisation. In *Tritonia* neurons the activation threshold is about -65mV and the channel conducts at potentials between -65 and -40mV (Thompson, 1977, Smith, 1978). At the resting potential of -45mV in these neurons the channels will be inactivated.

What is their role? In *Anisodoris* neurons after an action potential K_A channels are inactivated but delayed rectifier channels are active so that the cell hyperpolarises. This removes the inactivation of K_A channels and shuts down the I_K channels so the membrane begins to depolarize. This reactivates the K_A channels which open and the resulting outward current tends to keep the cell membrane potential negative for a period. Soon, however, the K_A channels inactivate and depolarisation increases until the firing threshold is again reached leading to the generation of another action potential. In this way these channels space successive action potentials. Such currents have been observed in arthropod muscle and neurons and in vertebrate neurons and the heart (for reviews see Rudy, 1988; Llinás, 1988).

The cardiac I_A is found specifically in Purkinje fibres and the atrium and makes up the I_{to} current. In human atria (Escande *et al.*, 1987) and in sheep Purkinje fibres (Coraboeuf and Carmeliet, 1982) two components of the current can be distinguished. One is long lasting (I_{lo} , l = long) and has a slow decay and is blocked by 4-AP and Ba^{2+} - the I_A component. The other is brief (I_{bo} , b = brief) and has a short rise time and decay. This component can be blocked by Co^{2+} and thus may be a Ca^{2+} -activated K^+ current.

4-AP blocks I_A with an IC_{50} of about 2mM in many different tissue types

(Rogawski, 1985). Since it is more potent on I_A than on $I_{K(Ca)}$ currents, 4-AP can be used to distinguish between them in some cells (Rudy, 1988). I_K and I_A can also be distinguished pharmacologically because I_A is not as sensitive to TEA and more sensitive to 4-AP than I_K (Connor and Stevens, 1971, Thompson, 1977).

1.1.3: THE M CURRENT (I_M)

This current was first discovered in frog sympathetic neurons (Brown and Adams, 1980). It was termed the M-current due to its inhibition by acetylcholine acting at muscarinic receptors. It displays time- and voltage- dependent characteristics. The current is activated slowly upon depolarisation, in 10-100ms, and inactivates slowly when the membrane is hyperpolarised. This channel regulates cell excitability in that it is inhibited by neurotransmitters such as acetylcholine, Substance P and luteinizing hormone releasing hormone (LHRH) which leads to an increase in excitability. These channels are found in both the periphery, for example in sympathetic neurons, and in many central neurons including human neocortical cells (Halliwell, 1986).

The neurotransmitters affecting the channel act via a G-protein which is pertussis toxin insensitive. The phospholipase C and the phosphoinositol pathway may be involved (Dutar and Nicoll, 1988a, b). Protein kinase C may be phosphorylating the channel as it inhibits some M current in certain tissues (Brown, 1988). The current is blocked by 1mM Ba^{2+} and by TEA (Constanti and Sim, 1987, Storm, 1989).

1.1.4: CALCIUM-ACTIVATED K^+ CHANNELS

These currents are activated by an increase in cytoplasmic free $[Ca^{2+}]$ and are variously termed $I_{K(Ca)}$, $I_{K, Ca}$, I_C , or I_{AHP} . With the introduction of patch clamp techniques, large conductance K(Ca) channels were discovered in nearly every vertebrate excitable cell. In addition to these large conductance channels, [(BK) or maxi-channels], intermediate conductance ($I_{K(Ca)}$) and small conductance channels ($S_{K(Ca)}$) also exist. These channels differ in their voltage-dependence, Ca^{2+} sensitivity, pharmacology and conductance.

BK channels were first discovered in chromaffin cells (Marty, 1981) and in rat myotubes (Pallotta *et al.*, 1981). They were observed to have large conductances - in equal external and internal K^+ (150mM) single channel studies show a conductance of between 150 and 250pS (Pallotta *et al.*, 1981). The channels are voltage-dependent and activate with depolarisation (Pallotta *et al.*, 1981; Moczydlowski and Latorre, 1983). The gating is also dependent upon the intracellular Ca^{2+} concentration (Barrett *et al.*, 1982). The activation by Ca^{2+} can occur with concentrations of between 0.2-10 μ M (Barrett *et al.*, 1982, Latorre *et al.*, 1982). These channels are responsible for rapid after-hyperpolarisations for example in frog spinal motoneurons (Barrett and Barrett 1976) and in superior cervical ganglion cells (Freschi, 1983, Kawai and Watanabe, 1986).

The BK channels are blocked by TEA at between 0.2 and 5mM. External TEA is more effective ($IC_{50} = 0.29$ mM) than internal TEA ($IC_{50} = 0.45$ mM) (Latorre, 1986). Charybdotoxin blocks the channels in the nanomolar range ($IC_{50} \sim 10$ nM) (Miller *et al.*, 1985) as does noxiustoxin ($IC_{50} \sim 450$ nM) and iberiotoxin.

SK channels have a smaller conductance of between 2 and 14pS. They display little voltage-dependence, and the gating is determined only by intracellular Ca^{2+} levels. Half maximal activation occurs at Ca^{2+} concentrations of 200-500nM (Blatz and Magleby, 1986). They are responsible for slow AHPs after an action potential in many neurons. During an action potential the internal Ca^{2+} concentration increases and this results in the activation of the slow AHP. This may slow the firing rate or even hyperpolarise the cell to the extent that the threshold potential cannot be reached so that action potentials stop - this is referred to as spike frequency adaptation.

Apamin is a potent blocker of many SK channels (Hugues *et al.*, 1982) and has been used to identify the current in e.g. rat superior cervical ganglion neurons (Kawai and Watanabe 1986) and guinea-pig hepatocytes (Burgess *et al.*, 1981). The channels are relatively insensitive to block by TEA (see Sah, 1996, for a review). The SK channel in cultured rat skeletal muscles is blocked by apamin but is insensitive to block by external TEA (Blatz and Magleby 1986).

A calcium-activated potassium conductance which produces prolonged AHPs in some tissues and is insensitive to apamin has also been discovered. These channels have been detected in rat hippocampal neurons (Lancaster and Nicoll 1987) and in peripheral neurons for example guinea-pig myenteric neurons (Kunze *et al.*, 1994). Both the apamin-sensitive and the apamin-insensitive SK channels have now been cloned (Kohler *et al.*, 1996).

The intermediate K(Ca) ($I_{K_{Ca}}$) was the first Ca^{2+} -activated K^+ conductance shown to exist and was discovered by Gardos in the 1950s. Activation occurs with $[Ca^{2+}]_i$ concentrations of 0.1-10 μ M (Hamill, 1983, Grygorczyk and Schwarz, 1983) as may occur as a result of depletion of intracellular ATP in red cells- the 'Gardos effect' (see the review by Gardos and Sarkadi, 1985). It is voltage-insensitive with a single channel conductance between the SK value and the BK value. In frog erythrocytes the conductance is 50pS in symmetrical K^+ (Hamill, 1983). Charybdotoxin blocks the erythrocyte channel as well as the BK channels (Wolff *et al.*, 1988). Quinine and quinidine block in submillimolar concentrations and cetiedil and oligomycin A are also effective (Armando-Hardy *et al.*, 1975, Berkowitz and Orringer, 1981, Riordan and Passow, 1971). These channels have now been cloned (Ishii *et al.*, 1997).

1.2: THE INWARDLY RECTIFYING POTASSIUM CHANNEL FAMILY.

The inward rectifier K^+ channels are so called because of reduced outward current flow at potentials positive to the reversal potential for K^+ , E_K , in comparison to the substantial inward current flow at potentials negative to E_K . Such behaviour was first described by Katz (1949) who referred to it as 'anomalous rectification' because it was in the reverse direction to the normal outward rectification seen with delayed rectifier currents.

These channels exhibit different degrees of rectification. Strong inward rectifiers such as the background inward I_{K_1} in cardiac tissue pass very little current at potentials positive to -40mV. This is relevant to their functions in maintaining a stable membrane potential during diastole and a long plateau in the action potential that are a common feature of the cardiac action potential in

many species. This current is also found in frog skeletal muscle, glial cells, and some CNS neurons.

Weak inward rectifiers also exist such as the K_{ATP} channel in the pancreatic β cells, some CNS neurons, the heart and all muscle cell types. These are termed weak rectifiers as there is substantial outward current flow at positive potentials. These channels can be modulated by somatostatin and galanin via a G-protein, for example in insulin secreting cell lines.

'Intermediate' inward rectifiers are present in various CNS neurons and in the heart. These channels are dependent on activation by receptor ligands, often through G-proteins. In cardiac tissue acetylcholine and adenosine modulate this K^+ channel, as is discussed in detail in a later section.

Figure 1.1 shows a cartoon representation of the current-voltage relationships for the three different inward rectifiers.

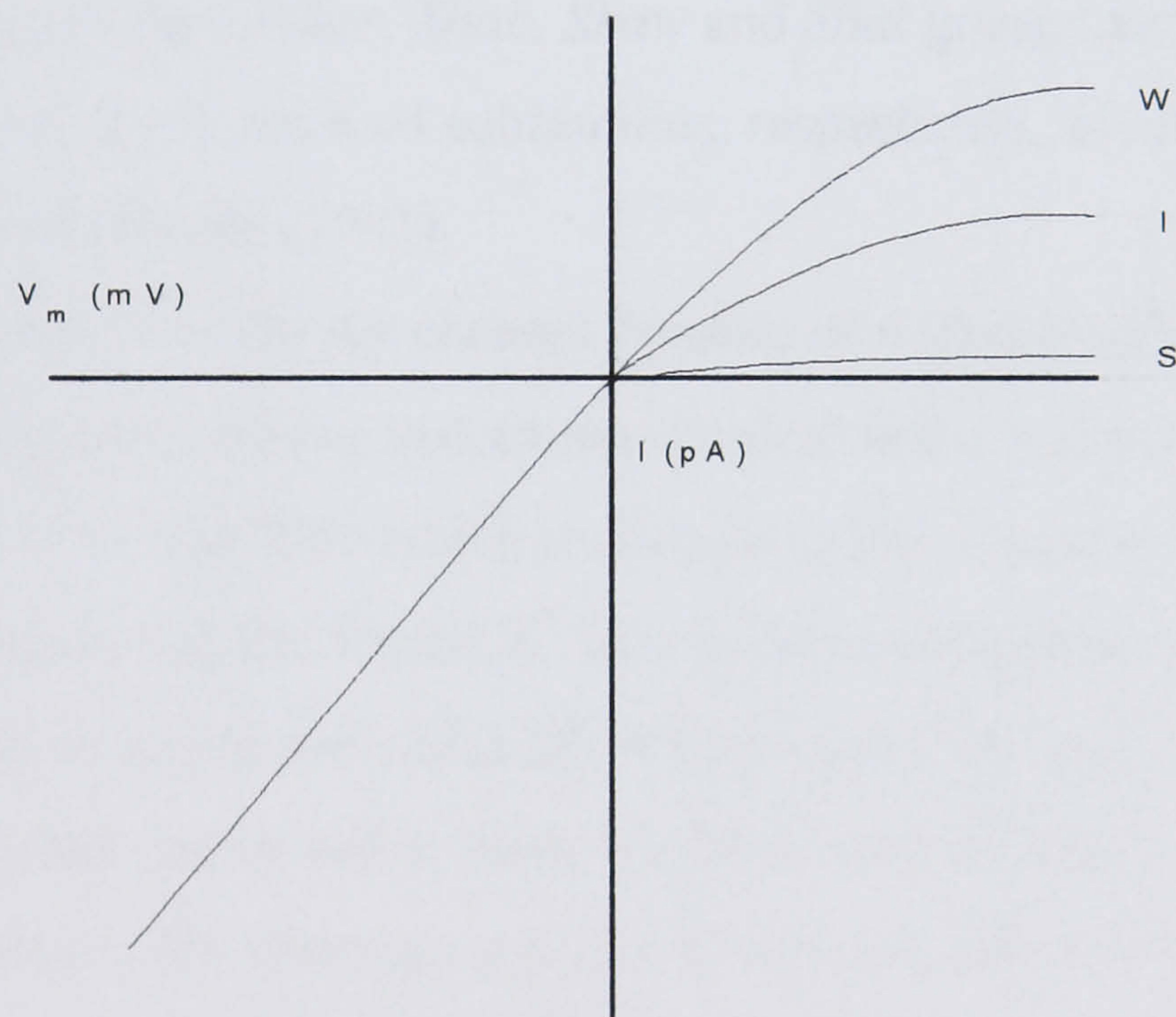


Figure 1.1: A schematic representation of the current-voltage relationships of the three main kinds of inward rectifier. In this hypothetical case E_K is set to 0 mV. Plots for strong (S), intermediate (I) and weak (W) inward rectifiers are illustrated.

The following sections deal with general aspects of the inwardly rectifying K⁺ channel family including their molecular biology, expression and the mechanism of rectification.

1.2.1: THE MOLECULAR BIOLOGY OF THE KIR FAMILY.

The cloning of the *Drosophila Shaker* locus led to the isolation and expression of a large family of voltage-dependent potassium channels, Kv (Papazian *et al.*, 1987, Tempel *et al.*, 1988). The Kv channel subunits are grouped into four subfamilies named after the *Drosophila* genes. In flies displaying the *Shaker* phenomenon of abnormal leg shaking in response to diethyl ether exposure, a transient outward-like K⁺ current was found to be missing from their leg and flight muscles (Salkoff 1985). Identification of the defective gene responsible for this led to the cloning of the first K⁺ channel (Tempel *et al.*, 1987, 1988). Using the *Shaker* gene as a probe allowed three related *Drosophila* K⁺ channels to be isolated (Butler *et al.*, 1989;1990). Together these are the *Shaker*, *Shab*, *Shaw* and *Shal* groups and are referred to as the Kv1, Kv2, Kv 3 and Kv4 subfamilies, respectively, according to the nomenclature of Chandy (1991)

The α -subunit of the Kv channel consists of 6 transmembrane domains (TM), a cytoplasmic carboxy and amino terminal and a hydrophobic 'P' or H5 loop between TM5 and TM6 which is thought to line the pore of the channel. Evidence suggests that the *Shaker* K⁺ channels are composed of four subunits so that the overall structure resembles the voltage-gated Na⁺ and Ca⁺ channels.

The tetramer can be either homo- or heteromeric (MacKinnon, 1991) though heteromeric Kv channels can be formed with subunits belonging to the same family only; subunits from different families fail to assemble. The assembly is thought to occur due to a specific interaction between the cytoplasmic N-terminal domains of the channel subunits (Shen *et al.*, 1995; Xu *et al.*, 1995). Heteromeric association between subunits may permit a large number of functionally similar Kv channel types to exist. A CNS Kv channel has been identified which comprises Kv1.2, Kv1.3, Kv1.4, Kv1.6 and Kv β 2 subunits and it is possible that Kv β 2, Kv1.2 and Kv1.4 may exist as homomultimers (Shamotienko *et al.*, 1997).

Homology screening of the Kv subunits did not lead to the identification of genes encoding inwardly rectifying K⁺ channels. In 1993 expression cloning of the cDNA and subsequent homology screening of two inwardly rectifying channels led to the discovery of a novel family of mammalian K⁺ channel genes. The two Kir channels were an ATP -dependent channel (Kir1.1a/ROMK1; Ho *et al.*, 1993) from the outer medulla of the mouse kidney and a classical K_{ir} channel (Kir2.1/IRK1; Kubo *et al.*, 1993) from a mouse macrophage cell line. These genes code for channel proteins distinct from the Kv family.

Kir channel cDNA was found to encode proteins of between 372 and 501 amino acids in length. The amino terminal is on the intracellular side and hydrophobicity plots reveal the presence of 2 transmembrane regions (TM1 and TM2). The TM1 and TM2 regions flank a highly conserved 'P' region which is similar to the region in Kv channels and may line part of the K⁺ selective channel pore (Jan and Jan, 1992; Brown, 1993; Pongs, 1993). These structures are all encoded for within the first half of the coding region. The carboxy end of the channel protein is also intracellular and there are thought to be a number of putative binding domains at the carboxy end as well as membrane associated domains. The Kir channel amino acid sequences diverge at the distal amino and carboxy termini and an extracellular loop linking TM1 and the P-region is also not very well conserved (Salkoff *et al.*, 1992).

By comparing the remaining 'core' sequences the presence of 6 Kir subfamilies was identified and these are designated Kir 1.0 to Kir 6.0 (Chandy and Gutman 1993). There is 40% sequence homology between subfamilies and 60% within a subfamily. The highest levels of sequence identity are found in the P-region and in the proximal carboxy end.

The subunit stoichiometry was examined for a background inward rectifier member, Kir2.1(IRK1) and it was shown that the channel is composed of four Kir2.1 subunits (Yang *et al.*, 1995) and a similar stoichiometry has been suggested for the G-protein-gated channels composed of members from Kir3 (GIRK) subunits (Inanobe *et al.*, 1995). The diversity of the channels is increased by subunits being able to form homomeric or heteromeric channels.

The G-protein gated channels in atrial cells of the heart are composed of Kir3.1(GIRK1) and Kir3.4(GIRK4) subunits and the channels in some of the brain neurons are composed of Kir3.1(GIRK1) and Kir3.2(GIRK2) (Krapivinsky *et al.*, 1995; Pessia *et al.*, 1996). The classical background inward rectifier is thought to be composed of homomeric Kir2.1 subunits (Kondo *et al.*, 1995).

The distribution of the different inward rectifiers and their roles are discussed in the following section.

1.2.2: SIX SUB-FAMILIES OF THE INWARDLY RECTIFYING POTASSIUM CHANNEL FAMILY: MOLECULAR AND FUNCTIONAL ASPECTS.

The naming of these channels is based on either the name given to the channel clone or the more uniform terminology proposed by Chandy and Gutman (1995) in which the subgroups are referred to as Kir1.x to Kir6.x. In this thesis the nomenclature of Chandy and Gutman will be adopted although sometimes the channel clone name is also given in parenthesis.

KIR 1:

Kir 1.1(ROMK1) is found mainly in the kidney and is a weak rectifier. It is both constitutively active and modulated by ATP for which there is a putative phosphate binding loop in the C-terminal end (Ho *et al.*, 1993). Its weak rectification is advantageous for a role in the export of K^+ into the collecting tubules of the distal nephron via the apical membrane of the renal epithelial cells.

The expression of Kir1.1 in the central nervous system remains unclear. Ho *et al.*, (1993) found it to be weakly expressed in the thalamus, hypothalamus and the pituitary. Others have found it to be expressed in the rat cortex and hippocampal neurons (Kenna *et al.*, 1994). In the human brain Kir1.1 mRNA has not been detected (Yano *et al.*, 1994; Shuck *et al.*, 1994).

KIR 2:

These channel types are all strongly inwardly rectifying and are important in setting the resting potential, buffering external K^+ , and modulating action potential waveforms without excessive K^+ loss during depolarisation. Three members have been cloned to date

The first channel of this type, Kir2.1, was cloned from a mouse macrophage cell line (Kubo *et al.*, 1993) and is expressed in heart ventricle and skeletal muscle. Kir2.1 is believed to be expressed throughout the brain, either in the glia or the vasculature (see review, Doupnik *et al.*, 1995). The channel has a single channel conductance of ~ 20 pS in 140mM $[K^+]$.

Kir 2.2 is found in the cerebellum in both mouse and rat brain and it is also present in rat kidney, heart and skeletal muscle (Takahashi *et al.*, 1994; Koyama *et al.*, 1994). The single channel conductance is ~ 35 pS (140mM external $[K^+]$).

Kir2.3 was cloned from human brain tissue (Perier *et al.*, 1994; Tang *et al.*, 1994; Makhina *et al.*, 1994) and from mouse and rat brain (Morishigue *et al.*, 1994; Lesage *et al.*, 1994; Bond *et al.*, 1994) and is expressed in the forebrain of all three species. It is also expressed in human and rat, but not mouse, heart. The single channel conductance is ~ 10 pS (140mM external $[K^+]$). The channel protein has a larger extracellular loop than the Kir2.1 and 2.2 being 15 amino acids longer between TM1 and the P-region (Bond *et al.*, 1994). The larger loop may be involved in membrane targeting or interaction with other membrane molecules.

The channels differ not only in their single channel conductance but also in their sensitivity to phosphorylation and other second messengers (Fakler *et al.*, 1994; Chang *et al.*, 1996; Henry *et al.*, 1996).

KIR 3:

These channels are regulated by G-proteins and are intermediate in rectification. Most work has been done on the channel expressed in the heart which was found to be composed of two Kir3 subunits, Kir3.1 (GIRK1) and Kir3.4 (CIR, for Cardiac Inward Rectifier) (Krapivinsky *et al.*, 1995). The

cardiac channel is activated by both acetylcholine and adenosine and by the $\beta\gamma$ subunits of activated G-proteins, as discussed in detail in a later section.

Kir3.1 has been found in some neurons of the mammalian brain and is thought to be involved in the suppression of neuronal firing. Two new members, Kir3.2 and Kir3.3, have since been cloned from the mouse brain (Lesage *et al.*, 1994).

Kir3.4 was cloned from the rat and human heart and is also expressed in brain tissue. It was initially thought to be identical to the native K_{ATP} channels when expressed in mammalian epithelial cell lines (Ashford *et al.*, 1994). It was subsequently shown, however, that Kir3.4 co-assembles with Kir3.1 to form the atrial G-protein-gated current (Krapivinsky *et al.*, 1995). Although Kir3.4 is not expressed in the pancreas, an isoform has been identified (Ashford *et al.*, 1994).

KIR 4 AND KIR 5:

These subfamilies are detected in the brain (Takumi *et al.*, 1995; Bond *et al.*, 1994) where Kir 4.1 and Kir5.1 have been shown to co-assemble to form novel channels. Kir4.1 is also expressed in the kidney (see Section 1.4.3.3). Kir 5.1 does not form functional channels when expressed in *Xenopus* oocytes (Bond *et al.*, 1994). These channels may co-assemble with other Kir channel subunits providing channel diversity (Pessia *et al.*, 1996).

KIR 6:

Kir1.1 was thought at first to form a constituent of K_{ATP} channels as the expressed channels are similar in properties to the endogenous kidney K_{ATP} . However the evidence for ATP_i inhibition of Kir1.1a (ROMK1) is inconclusive (Ho *et al.*, 1993; Lu and MacKinnon 1994; Nichols *et al.*, 1996). Based on sequence homology a novel ubiquitously expressed gene (u K_{ATP} or Kir 6.1, Inagaki *et al.*, 1995) was discovered which led to the discovery of a pancreatic isoform, Kir6.2 (BIR, for β -cell inward rectifier family). These subunits when co-expressed with a sulphonylurea receptor (see section 1.4.3.2) led to expression of the active K_{ATP} channel (Aguilar-Bryan *et al.*, 1995; Ammala *et al.*, 1996.)

Kir6.2 has been shown to form the pore of the channel, as with other Kir channels, and it is the sulphonylurea receptor which provides some of the nucleotide sensitivity (Nichols *et al.*, 1996). Further work has made clear that there is no SUR-independent activity of Kir6.2 and a close physical association occurs between the SUR and Kir6.2 (McNichols *et al.*, 1996; Schwanstecher *et al.*, 1994). Kir6.2 is strongly expressed in pancreatic β -cells, brain, heart, and skeletal muscle (Sakura *et al.*, 1995).

1.3: THE MODE OF INTRINSIC RECTIFICATION:

The following sections deal with the role of internal cations, intracellular factors and structural determinants which are all involved in inward rectification.

1.3.1: BLOCK BY INTERNAL CATIONS:

It was originally proposed that rectification may be due to block of the channel by a positively charged compound from the cytoplasmic side of the membrane in a voltage-dependent manner (Armstrong, 1971). Work done on the K_{ATP} channels revealed that both Mg^{2+} and Na^+ were able to support inward rectification (Ciani *et al.*, 1988; Horie *et al.*, 1987).

Matsuda *et al.*, (1987) used improved patch clamp techniques and showed that in adult guinea-pig ventricular I_{K_1} channels, outward single channel currents from cell attached patches were blocked by physiological levels of Mg^{2+} . This block was affected by voltage, increasing with depolarization, and the results suggested an open channel block by intracellular Mg^{2+} (Matsuda *et al.*, 1987; Shioya *et al.*, 1993; Martin *et al.*, 1995). The importance of Mg^{2+} in inward rectification was also shown for the G protein-gated K^+ current in guinea-pig atrial cells. Inside-out patches displayed outward current in Mg^{2+} -free solution after activation of the channel with 0.1 mM GppNHp applied to the inner surface of the membrane. Increasing the Mg^{2+} concentration reduced the unit amplitude of the outward currents and again the block increased with depolarization (Horie & Irisawa, 1987).

Vandenberg (1987) independently showed that the excision of inside-out ventricular myocyte patches into divalent cation-free internal solution resulted in the elimination of rectification of the single channel current. Subsequent addition of Mg^{2+} to the internal solution restored rectification.

Studies have also been carried out on cloned channels. Stanfield *et al.*, (1994) expressed the Kir2.1(IRK1) gene in murine erythroleukaemia cells which gave rise to channels which displayed inward rectification. Removal of Mg^{2+} from the internal solution led to the appearance of outward currents at depolarised potentials and the current-voltage relationship no longer displayed inward rectification. Expression of Kir1.1(ROMK1) in oocytes has demonstrated how the IC_{50} value for Mg^{2+} decreases with depolarization (Nichols *et al.*, 1994) and it was observed that the block by Mg^{2+} was rapid and weak, resembling that of K_{ATP} channels.

Later work has shown that the Mg^{2+} -dependent inward rectification is voltage-dependent, concentration-dependent and reduced by increasing extracellular K^+ ions, for example I_{K_1} in guinea-pig heart cells (Matsuda *et al.*, 1987, Taglialatela *et al.*, 1994), K_{ATP} channels in oocytes (Nichols *et al.*, 1994) and the atrial G-protein gated current (Horie and Irisawa, 1987). It is thought that increasing the extracellular $[K^+]$ 'knocks-off' Mg^{2+} from sites inside the pore thus decreasing the rectification. Also the repulsive effect of K^+ ions in the pore decreases the affinity for binding of Mg^{2+} and relieves the block (Armstrong 1971, Hille & Schwartz 1978, Horie *et al.*, 1987).

1.3.2: BLOCK BY INTRACELLULAR POLYAMINES.

Although the rectification process can be explained by an action of Mg^{2+} , there is much evidence to suggest that this is not the only factor. Strongly rectifying channels from a cloned inward rectifier displayed substantial rectification in the absence of Mg^{2+} in outside-out patches (Stanfield *et al.*, 1994). In guinea-pig cardiac myocytes the Mg^{2+} blocked channels transit to the closed state at maintained depolarised potentials, suggesting an intrinsic gating mechanism (Ishihara *et al.*, 1989). Excision of inside-out membrane patches from *Xenopus* oocytes containing cloned Kir2.3 (HRK1) channels resulted in

the loss of rectification if moved away from the surface of the cell (Lopatin *et al.*, 1994). In Mg^{2+} -free solution rectification was restored by moving the patch back towards the surface of the cell. It was concluded that intrinsic factors were being released from oocytes and causing rectification. In bovine pulmonary artery endothelial cells rectification is observed both in the absence and presence of internal Mg^{2+} (Silver and DeCoursey 1990) and a Kir2.1 channel expressed in the mouse fibroblast cell line also displayed both Mg^{2+} -dependent and Mg^{2+} -independent rectification (Ishihara *et al.*, 1995).

Subsequent biochemical analysis of the cytosol of oocytes and other cells revealed that the cytoplasmic factors involved were small positively charged molecules - polyamines (Lopatin *et al.*, 1994). Polyamines are naturally occurring and are present in cells in mM levels. The naturally occurring polyamines such as spermine, spermidine and putrescine restore all the features of rectification when applied to inside-out patches containing Kir2 channels (Matsuda *et al.*, 1988; Ishihara *et al.*, 1989; Lopatin *et al.*, 1994, 1995; Fakler *et al.*, 1994; Ficker *et al.*, 1994). The voltage dependencies of the block by polyamines are steeper than for Mg^{2+} (Fakler *et al.*, 1994; Ficker *et al.*, 1994; Lopatin *et al.*, 1994, 1995). This explains why the rectification seen in situ is steeper than predicted for Mg^{2+} alone (Hille 1992).

The degree of rectification is thus due to the extent of block by Mg^{2+} and polyamines. High affinity polyamine and Mg^{2+} block is seen with strongly rectifying channels. Kir1.1 (ROMK1) channels show mild inward rectification and channels expressed in oocyte patches did not strongly rectify when brought to the surface of an oocyte. Application of oocyte-conditioned internal medium (oocytes were incubated for up to 3 hours in K^+_{int} solution, the solution was then aspirated and filtered, and stored at 4°C) did not lead to strong rectification, as is the case with IRK1 (Lopatin *et al.*, 1994).

Subsequent work has shown that Mg^{2+} and polyamines may share the same binding site within the channel (see below). Site-directed mutagenesis studies also revealed that the structure of the channel plays a part in dictating the degree of rectification observed (Pessia *et al.*, 1995; Taglialatela, *et al.*, 1994).

The following section discusses the contribution of the channel structure to the rectification process.

1.3.3: STRUCTURAL DETERMINANTS OF INWARD RECTIFICATION.

Studies have shown that the TM2 region forms part of the lining of the pore (Fakler *et al.*, 1994; 1995; Ficker *et al.*, 1994; Matsuda *et al.*, 1994; Taglialatela *et al.*, 1994) and that the different sensitivities of the inward rectifiers to block by Mg^{2+} and polyamines could be attributed to differences in their structure at this region. Stanfield *et al.*, (1994) showed that an exchange of aspartate at position 172 in the TM2 region for glutamine in murine Kir channels resulted in a five-fold reduced affinity for Mg^{2+} and weaker inward rectification. The negative charge carried by aspartate is important for voltage-dependent channel gating and may play a part in the binding of Mg^{2+} .

This has been observed in other inward rectifier channels. In Kir2.1(IRK1) the amino acid at position 172 is aspartate and in Kir1.1(ROMK1) it is asparagine at position 171. Mutation of the aspartate to asparagine in Kir2.1 leads to Kir1.1 like gating and conversely mutating the asparagine in Kir1.1 to aspartate leads to Kir2.1 type gating (Wible *et al.*, 1994). The amino acid at position 171 in Kir1.1 (ROMK1) is an asparagine and substitution for an aspartate - found in Kir2.1 and Kir3.1- converts the weak Kir1.1 to a strong rectifier (Lu and MacKinnon 1994). In Kir6.2, the equivalent amino acid in TM2 at residue 153 is an asparagine and the channel displays weak rectification (Inagaki *et al.*, 1995). Mutation of the asparagine residue in the TM2 region at position 160 to aspartate increased the degree of rectification by both Mg^{2+} and polyamines in cloned Kir6.2 K_{ATP} channels (Shying *et al.*, 1996).

It was also shown that the potency of spermine for the different inward rectifier channels- Kir2.1(IRK1), Kir4.1(BIR10) and Kir1.1 (ROMK1)- was strongly influenced by the particular amino acid residue at positions analogous to 171/172 (Fakler *et al.*, 1994). The block of an outward current at potentials positive to E_K by spermine was measured for all three subtypes and fitting the Hill equation gave IC_{50} values of 31nM, 40nM, and 0.78mM for Kir2.1, Kir4.1 and Kir1.1 respectively at a membrane potential of +50mV. A mutation at

position 158 in the TM2 region of BIR10 from glutamate to asparagine decreased the spermine affinity and the IC_{50} value increased from 40nM in the wild type BIR10 to 3.5mM, a loss of 5 orders of magnitude in sensitivity (Fakler *et al.*, 1994).

The C-terminal domain is also important for inward rectification. A chimera was constructed in which the C-terminal domains from Kir3.1(GIRK1) and Kir6.2 (BIR) were exchanged. The chimera which had the C-terminal domain from Kir3.1(GIRK1) did not need G-protein activation, displayed activation and deactivation kinetics and rectification properties similar to those of the Kir3.1(GIRK1) channel. This shows that structural elements in the C-terminal can independently control gating and that gating is not solely dependent on the residue in the TM2 region as in this chimera the residue in TM2 was from BIR10 yet rectification was of the GIRK1 type. (Pessia *et al.*, 1995). Similarly chimeras between the weakly rectifying Kir1.1(ROMK1) and the strongly rectifying Kir2.1(IRK1) suggests that the C-terminal contains structural elements which are needed for strong rectification and high Mg^{2+} affinity, even though the rectification residue in the TM2 region was not exchanged (Taglialatela *et al.*, 1994). In the C-terminal domain of Kir2.1 glutamic acid at position 224 is an important determining factor of Mg^{2+} and polyamine sensitivity as neutralization of this residue and the negative charge in TM2 (172) reduces the Mg^{2+} and polyamine affinity of Kir2.1 to that of Kir1.1(ROMK1) (Yang *et al.*, 1995).

The off-rates of different polyamines and Mg^{2+} from the channel are influenced by the amino acid residue at position 84 preceding the TM1 region in Kir2.1 channels (Ruppersberg *et al.*, 1996). The release of Mg^{2+} from Kir2.1 is reported to occur with a half time ($t_{1/2}$) of 10.2 ± 0.4 ms and spermine release with a $t_{1/2}$ of 1.6 ± 0.3 ms at -50mV (Ruppersberg *et al.*, 1996). Kubo *et al.*, (1993) observed a $t_{1/2}$ for Mg^{2+} release of 0.1 ± 0.05 ms and for spermine release of 0.3 ± 0.1 ms in a different Kir2.1 channel. The difference was attributed to the presence of threonine at position 84 in the Ruppersberg clone and a methionine in the Kubo clone. The residue in Kir1.1 is lysine and in Kir3.4 it is asparagine and mutation of threonine in Kir2.1 to a lysine revealed a fast release of both

Mg²⁺ and spermine ($t_{1/2}$ of less than 0.1 ms) and mutation of the threonine to asparagine resulted in a slow release, $t_{1/2}$ of ~49 ms for Mg²⁺ and ~24 ms for spermine (Ruppersperg *et al.*, 1996).

Thus it appears that Mg²⁺ and polyamines can interact with at least two negatively charged amino acid sites one of which is in the TM2 domain at positions analogous to 171/172 [receptor site 1 (R1)] and the other in the C-terminus at positions analogous to 224 [receptor site 2 (R2)]. For strong rectifiers the amino acids both carry negative charges whilst in the G-protein gated channels only one of the sites is negative and for the weak rectifiers they are both neutral.

Chimeras between Kir3.1(GIRK1) and Kir1.1a(ROMK1) indicate that there are residues in TM1 and TM2 regions which affect the strong rectification seen in the Kir3.1 channel. The intermediate rectification of the G-protein-gated channel was reduced if the acidic aspartate residue at 173 was mutated to glutamine (analogous to position 171, Lu and MacKinnon 1994)). Also residues in the P region pore-forming domain are important in the Kir3.1 time-dependent activation. A phenylalanine at position 137 is unique in Kir3.1 and it seems to be important in the time-dependent gating. G-protein activation of the channel is not affected by mutations in the TM1 and TM2 regions. (Kofuji *et al.*, 1996).

The amino acids present at R1 and R2 and in the H5/P-region are shown in Figure 1.3.3.1. The inward rectifiers have a G-Y-G sequence within the H5 region which forms the K⁺-selective pore (MacKinnon, 1991; Yang *et al.*, 1996). Figure 1.3.3.2 shows a schematic diagram of the channel illustrating the important residues involved in rectification as mentioned above. Also shown is the evolutionary tree for the inward rectifiers.

A: Pore sequences

133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
GIRK1																	
A	F	L	F	F	I	E	T	E	A	T	I	G	Y	G	Y	R	Y
ROMK1																	
				S	L			Q	V						F		F
IRK1																	
				S				Q	T						F		C
GIRK2																	
				S				E							Y		V

B: Mg²⁺ and polyamine binding sites:

	R1	R2
Kir1.1(ROMK1)	N	G
Kir2.1-2.3(IRK1-3)	D	E
Kir3.1(GIRK1)	D	S
Kir3.2-3.4(GIRK3-4)	N	E
Kir4.1(BIR10)	E	G
Kir6.1(uKATP-1)	N	S
Kir6.2(BIR)	N	S

Figure 1.3.3.1: A, the amino acid residues in the pore sequence of four different inward rectifiers (from Koufuji *et al.*, 1996). B, the amino acid residues present in the R1 - M2 domain- and the R2 - C-terminal domain. The Kir2 family are strong rectifiers and have negative residues at M1 and M2. Kir6 members are weak and have the neutral residues at these positions. Kir1.1 and Kir4.1 have an ATP-binding domain within their C-terminals. These two positions are thought to be the binding sites for Mg²⁺ and polyamines (Isomoto *et al.*, 1997). The amino acid key is: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; N, asparagine; Q, glutamine; S, serine; T, threonine; V, valine; Y, tyrosine

A: Image removed due to third party copyright

Image removed due to third party copyright

B:

Figure 1.3.3.2: A: Cartoon representation of the Kir structure, taken from Nichols & Lopatin 1996. The GYG sequence in the pore is conserved in Kv channels and is thought to form the K^+ selective pore. Position 171 in the M2 region and 224 in the C-terminus are shown. The Mg^{2+} off rates are thought to be controlled by an amino acid around position 84. B: The evolutionary tree of the inward rectifier family. Members of the same family are ~ 50-70% identical, whilst members of different families are ~ 40% identical. (Isomoto *et al.*, 1997).

1.4.1: THE ATRIAL G-PROTEIN GATED K^+ CHANNEL.

Parasympathetic stimulation decreases the heart rate by the activation of muscarinic receptors and the subsequent opening of K^+ channels in the sinoatrial node and the atrium in the heart. These channels display intermediate inward rectification, and in contrast to I_{K_1} , there is substantial outward current flowing at depolarized potentials. The single channel conductance is approximately 50pS in high external $[K^+]$ (Sakmann and Trube 1984; Kurachi *et al.*, 1985; Ito *et al.*, 1992).

Both acetylcholine and adenosine can activate the K_{ACh} channel, hyperpolarise the myocyte and reduce the action potential duration (Kurachi *et al.*, 1986; Belardinelli & Isenberg 1983). The involvement of G-proteins has been shown for example in guinea-pig atrial myocytes where the responses to both agonists required the presence of GTP on the intracellular side of the membrane; also, the conductance and kinetics of the whole cell and single channel currents indicated that the two agonists activated the same population of K^+ channels (Kurachi *et al.*, 1986). Again in ferret ventricular myocytes both agonists activated a single population of inwardly rectifying channels which are distinct from the background inward rectifier channels (Ito *et al.*, 1995). All the evidence indicates that acetylcholine and adenosine activate the same population of K^+ channels via G-protein activation

1.4.1.1: G-PROTEIN REGULATION OF THE K_{ACh} CHANNEL.

Much work has gone into defining the involvement of G-proteins with the cardiac G-protein gated K^+ channels. It has been shown that the activation of the K_{ACh} channel is mediated by a pertussis toxin-sensitive G-protein via a direct membrane delimited pathway with no involvement of second messengers (Breitwieser & Szabo 1985; Pfaffinger *et al.*, 1985; Ito *et al.*, 1992;). The G-protein involved in the activation of $I_{K_{ACh}}$ was initially designated G_K due to its function of activating a K^+ conductance (Breitwieser and Szabo 1985) but is now generally referred to as G_i . The inactivation of GTPase, and thus the G-protein, within chick atrial cells by ADP ribosylation using pertussis toxin results in the prevention of any current activation in response to the application of acetylcholine (Pfaffinger *et al.*, 1985). In inside-out patches the activation of

the current by acetylcholine and adenosine required GTP on the cytoplasmic face and the activation was inhibited by the A promoter of pertussis toxin providing further evidence for the involvement of a G-protein (Kurachi *et al.*, 1986). Intracellular dialysis of GTP- γ -S into guinea-pig atrial myocytes increased the $I_{K_{ACh}}$ in the absence of any agonist and pretreatment with pertussis toxin prevented the increase (Ito *et al.*, 1991). It has also been observed that in cell attached membrane patches the presence of ACh in the pipette but not in the bathing solution activates the channel (Soejima and Noma 1984). This evidence suggests that the activation of the current is membrane delimited rather than indirectly via second messengers.

Since the discovery of G-protein activation of $I_{K_{ACh}}$ many groups have speculated about the exact mechanism of activation of the channel by the G-protein subunits and it has been debated as to whether it is G_α or $G_{\beta\gamma}$ which activates the channel. Clapham and coworkers (Logothetis *et al.*, 1987, 1989; Clapham and Neer, 1993) postulated that the $\beta\gamma$ subunits are responsible. Studies done by this group showed that the K^+ channel in chick embryonic atrial cells could be directly activated by the addition of purified $\beta\gamma$ subunits from bovine cerebral cortex to the inner surface of an inside-out patch. (Logothetis *et al.*, 1987). Another study showed that $\beta\gamma$ subunits isolated from rat brain could activate the guinea-pig atrial K_{ACh} in inside-out patches (Kurachi *et al.*, 1989). $\beta\gamma$ subunit inhibitors derived from β -adrenergic receptor kinase 1 decreased channel opening probability and increased closed times in inside-out patches from rabbit atrial myocytes (Nair *et al.*, 1995) providing further evidence for $\beta\gamma$ activation of K_{ACh} .

These observations were criticized by Brown, Birnbaumer and their coworkers on the grounds that the detergents used to suspend the $\beta\gamma$ -subunits could themselves activate the channels and that the $\beta\gamma$ -subunits prepared from the brain might be contaminated by α -subunits (Yatani *et al.*, 1987) which it was suggested were responsible for K_{ACh} activation. This was effectively ruled out by applying the $\beta\gamma$ subunits in Mg^{2+} -free solution which would have inactivated any G_α present but did not affect the $\beta\gamma$ subunit activation of the

channel. Also the application of the detergent CHAPS alone did not result in the activation of the channel (Kurachi *et al.*, 1989). It was also shown that the activation of the channel by recombinant $\beta\gamma$ -subunits or GTP- γ -S can be reversed by α -GDP (Logothetis *et al.*, 1988). This study ruled out any possible contamination in the preparations or any artifactual activation by the detergents used to solubilize the $\beta\gamma$ -subunits. Yamada *et al.* (1994) also demonstrated the activation of the channel by $\beta\gamma$ -subunits and inhibition by α -GDP.

Schreibmayer *et al.*, (1996) observed that GTP- γ -S activated $G\alpha$ actually inhibits $G\beta\gamma$ activation of K_{ACh} in excised membrane patches of *Xenopus* oocytes expressing the channel. Many other studies have also pointed to the activation of K_{ACh} by $\beta\gamma$ subunits (see Kurachi *et al.*, 1989; Ito *et al.*, 1991; Reuveny *et al.*, 1994; Yamada *et al.*, 1993; and Kurachi 1995; Muller and Lohse 1995; Wickman and Clapham 1996 for reviews).

A contrasting view was proposed by Brown and Birnbaumer's group whose results indicated that it was the α subunit which activated the K_{ACh} whilst $\beta\gamma$ did not (Yatani *et al.*, 1987) (Codina *et al.*, 1987). In addition a monoclonal antibody against $G\alpha$ blocked the carbachol induced stimulation of K_{ACh} (Yatani *et al.*, 1988a) and it was also observed that three recombinant forms of the α -subunits of G_i activated the K^+ channel (Yatani *et al.*, 1988b). Hence, Brown, Birnbaumer and their group concluded that $G\alpha$ activated the K^+ channel. However, this work could not be reproduced by Clapham and coworkers (Nanavati *et al.*, 1990). It has also been demonstrated that purified $G\beta\gamma$ from bovine brain acts through the same molecular mechanism as the endogenous G_i in guinea-pig atrial myocytes (Yamada *et al.*, 1993). So it would appear that $G\alpha$ is not responsible for activation of $I_{K_{ACh}}$ and the view that $G\beta\gamma$ activates the muscarinic K^+ channel has become widely accepted.

The involvement or otherwise of second messengers in the activation of $I_{K_{ACh}}$ by $G\beta\gamma$ has also been debated. It was reported that inhibitors of lipoyxygenase or antibodies which block PLA_2 activity inhibited the activation of K_{ACh} by $G\beta\gamma$ (Kim *et al.*, 1989a) although it was also observed that some inhibitors of lipoyxygenases and PLA_2 did not block $G\beta\gamma$ activation (Ito *et al.*,

1992). It was also found that metabolites of arachidonic acid could activate $I_{K_{ACh}}$ (Kim *et al.*, 1989b, Kurachi *et al.*, 1989) though the activation by the arachidonic acid metabolites was shown to be weak compared with the $G\beta\gamma$ activation (Kim *et al.*, 1989b)

Recently it has been shown that $\beta\gamma$ binds directly to the N-terminal hydrophilic domain and the amino acids 273-426 in the C-terminal domain of Kir3.1(GIRK1) (Huang *et al.*, 1995; Slesinger *et al.*, 1995). This direct binding of the $\beta\gamma$ subunit to the C-terminal has also been demonstrated in mouse Kir3.1(GIRK1) (Inanobe *et al.*, 1995). It was well established by this stage that K_{ACh} is a heteromultimer of Kir3.1 (GIRK1) and Kir3.4 (CIR) (Krapivinsky *et al.*, 1995a) and further experiments showed that the $\beta\gamma$ -subunits bind not only to the Kir3.1 subunit but also to Kir3.4 (Krapivinsky *et al.*, 1995b). The binding of $\beta\gamma$ to immuno - precipitated K_{ACh} and recombinant Kir3.1 and Kir3.4 had dissociation constants (K_d) of 55, 50 and 120nM respectively and it was also demonstrated that $G\alpha$ did not bind to the subunits at a detectable level. Chimeric studies replacing the C-terminus of Kir3.1(GIRK1) with that of Kir2.1(IRQ1) resulted in persistent activation of the chimera. Conversely, replacement of the C-terminal of the Kir2.1 with the C-terminal of Kir3.1 allowed $G\beta\gamma$ to activate Kir2.1 (Takao *et al.*, 1994). Chimeras between Kir3.1 and Kir3.3 have led to the identification of the important regions in channel regulation by G-proteins. There are regions in the N-terminal domain and the C-terminal domain of the Kir3.1 which are both capable of binding $\beta\gamma$ in a chimeric channel (Kunkel and Peralta, 1995). Expression of Kir3.1 and Kir3.4 in *Xenopus* oocytes has demonstrated that the N- and the C-termini of both subunits have G protein sensitivity and both are needed for maximal stimulation by cloned $G\beta_1\gamma_2$ (Tucker *et al.*, 1996).

The physiological characteristics of G-protein activation of K_{ACh} have also been studied. In inside-out patches from guinea-pig atrial myocytes (Ito *et al.*, 1991), increasing the concentration of ACh in the pipette resulted in a decrease in the threshold concentration of intracellular GTP ($[GTP]_i$) needed to activate the channels. A decrease in the EC_{50} value of GTP and an increase in the maximum response were also observed (seen as an increase in the product of the

number of active channels and their open probability). A Hill coefficient of ~ 3 has been estimated for GTP_i activation of the channel; altering the concentration of ACh did not affect this value (Ito *et al.*, 1991; Kurachi *et al.*, 1990; Yamada *et al.*, 1994). As the functional K_{ACh} is composed of 4 channel subunits it is possible that $G\beta\gamma$ binds to individual subunits resulting in positive cooperativity.

1.4.1.2: MOLECULAR BIOLOGY OF THE K_{ACh} CHANNEL

Kir3.1 (GIRK1) mRNA was first isolated from the rat atrium and when coexpressed with the muscarinic receptor in *Xenopus* oocytes results in inwardly-rectifying currents in response to the application of acetylcholine (Kubo *et al.*, 1993; Dascal *et al.*, 1993). Whilst the Kir3.1 expression results in currents similar to the native $I_{K_{ACh}}$, there are some differences. For example, there is weak stimulation by G-proteins and high agonist-independent activity. It was thus thought that the Kir3.1 was not the only subunit needed for the expression of the K_{ACh} channel. Subsequent work with antibodies specific for Kir3.1 showed that immunopurification of the Kir3.1 protein from bovine heart resulted in the purification of a second protein (Krapivinsky *et al.*, 1995). Sequence analysis showed that this second protein was a previously cloned inward rectifier termed CIR (Cardiac Inward Rectifier) or Kir3.4 and this was found to be almost identical to the rcK_{ATP-1} cloned by Ashford *et al.*, (1994) which was thought to belong to the K_{ATP} family. It was concluded that the cardiac G-protein gated K^+ channel is composed of Kir3.1 and Kir3.4. rcK_{ATP} therefore did not belong to the K_{ATP} family but to the G-protein-gated family of inward rectifiers (GIRK).

A mouse cDNA library was used to identify two additional homologues of Kir3.1, Kir3.2 (GIRK2) and Kir3.3 (GIRK3) (Lesage *et al.*, 1994). Alternative splicing of the gene encoding mouse Kir3.2 generates three isoforms, termed Kir3.2A (GIRK2A), Kir3.2B (GIRK2B) and Kir3.2C (GIRK2C) (Isomoto *et al.*, 1996). These spliced variants have a common N-terminal and core region though they differ at their C-terminal ends. GIRK2B was found to be ubiquitously distributed (Isomoto *et al.*, 1996). Channels composed of these subunits are important in the CNS.

1.4.1.3: THE G PROTEIN-REGULATED K^+ CHANNEL IN THE BRAIN.

G protein coupled channels in the brain suppress neuronal firing. Kir3.1 is expressed in some CNS neurons (see Doupnik *et al.*, 1995). Two members cloned from mouse brain are Kir3.2 and Kir3.3 (Lesage *et al.*, 1994) and may contribute to the diversity in neuronal G protein-activated currents. Kir3.2 when expressed in *Xenopus* oocytes produces receptor-evoked Kir3.1-like currents. Kir3.3 subunits do not produce receptor activated currents or constitutively active current and are thought to co-assemble with other Kir3.x subunits producing diversity within the group (Andrade *et al.*, 1986; North 1989; Chen *et al.*, 1993).

Many neurotransmitters in the brain activate an inwardly rectifying K^+ conductance via a G-protein, for example GABA acting at GABA_B receptors in hippocampal pyramidal cells (Bowery, 1980, Duttar and Nicoll, 1988), adenosine at A1 receptors in CA1 pyramidal neurons in rat hippocampal slices (Greene and Haas, 1985; Trussel and Jackson, 1987), acetylcholine at M2 receptors (Egan and North, 1986), dopamine at D2 receptors in rat substantia nigra (Lacey *et al.*, 1987), noradrenaline at $\alpha 2$ receptors in hippocampal pyramidal cells (Madison *et al.*, 1987), serotonin at 5HT-1A receptors (Andrade *et al.*, 1986) and opiates at μ receptors (North *et al.*, 1987). In summary these act at the forebrain regions of the hippocampus, thalamus, striatum and the neocortex, in the midbrain regions of the raphe nucleus and the substantia nigra and in the hindbrain regions of the locus coeruleus, pontine, reticular nucleus and the parabrachial nucleus.

1.4.2: THE BACKGROUND INWARDLY RECTIFYING POTASSIUM CHANNEL.

'Background' inward rectifiers (I_{K_1}) were first discovered by Katz in K^+ depolarised muscle (1949) and they have been investigated particularly in frog skeletal muscle (Standen and Stanfield, 1978, 1979), starfish eggs (Hagiwara *et al.*, 1978) and tunicate eggs (Ohmori *et al.*, 1978).

The background inward rectifier has been studied in great detail in cardiac preparations. The I_{K_1} conductance in Purkinje fibres, ventricular and atrial tissue

is the dominant component of the resting membrane conductance (Ber-Heler and Reuter 1970; Kurachi 1985). More current flows in the inward direction at potentials negative to E_K than outward current at potentials positive to E_K . The low conductance at the plateau potential of the action potential helps to maintain the plateau and minimizes the loss of K^+ from the cell during depolarisation.

The first inward rectifier, Kir2.1(IRK1) to be cloned by the use of expression cloning techniques was in a mouse macrophage cell line (Kubo *et al.*, 1993). Three kinds of I_{K_1} have since been cloned from the mouse brain and have been designated MB-IRK1(Kir2.1), MB-IRK2 (Kir2.2) and MB-IRK3(Kir2.3) (MB for mouse brain; Takahashi *et al.*, 1994; Morishige *et al.*, 1993; 1994). mRNA for Kir2.1(IRK1) has been found in the heart, the brain and skeletal muscle. Expression in *Xenopus* oocytes leads to a strongly inwardly rectifying current which is blocked by extracellular Ba^{2+} and Cs^+ in a voltage- and concentration-dependent manner. The decay of the current following activation by hyperpolarization is more prominent for Kir2.2 than Kir2.1 and Kir2.3 and is thought to be due to block by extracellular cations such as Na^+ (Ohmori *et al.*, 1978; Biermans *et al.*, 1987; Harvey and Ten Eick, 1989; Kelley, 1992; Voets *et al.*, 1996). The single channel conductance of Kir2.1, Kir2.2 and Kir2.3 are ~ 22 , ~ 34 , and ~ 13 pS respectively in 150mM $[K]_o$. The cardiac I_{K_1} has a single channel conductance of ~ 40 pS, with 150mM $[K^+]_o$. There is voltage-dependent inactivation of this current during maintained depolarisation in the absence of any blocking cations which is thought to be due to intrinsic inactivation (Sakmann & Trube 1984; Kurachi 1985). This is similar to the properties of Kir2.2 (Takahashi *et al.*, 1993) and the I_{K_1} channel in heart is thus thought to be composed of homotetrameric Kir2.2 subunits.

1.4.2.1: TWIK-1

This is a weak inward rectifier of 336 amino acids in length and was the first in a group isolated from a human kidney cDNA library (Lesage *et al.*, 1996). The hydrophobicity plot revealed 4 transmembrane domains and it has 2 pore motifs in tandem. Assuming that 4 pore domains are needed for a functional K^+ channel it is expected that TWIK-1 channels will prove to be

dimers. It does not possess a voltage-sensing domain and it is a weak inward rectifier having a single channel conductance of 34pS in symmetrical K^+ and showing Mg^{2+} -dependent rectification. The currents seen on expression in *Xenopus* oocytes are blocked by Ba^{2+} ($IC_{50} = 100\mu M$) and class I antiarrhythmics, such as quinine ($IC_{50} = 50\mu M$) and quinidine ($IC_{50} = 95\mu M$). According to Northern analysis the mRNA is widely distributed in human tissues especially in the brain and heart. These channels may be involved in the control of the background conductance, the extent of which has yet to be determined.

1.4.3: THE ATP-SENSITIVE K^+ -CHANNEL

K_{ATP} channels were first discovered in the heart (Noma 1983) where they were observed to be blocked by ATP_i above 1mM and to have a single channel conductance of 80pS in 100mM K^+ . ATP-sensitive channels have since been shown to be involved in the shortening of the action potential duration and in the cellular loss of K^+ observed during metabolic inhibition. They open in response to metabolic inhibition or hypoxia and thus couple the metabolic state of the heart to the membrane potential. They are found in a wide variety of tissues such as pancreatic β -cells, skeletal muscle, smooth muscle, and some neurons of the central nervous system.

As noted earlier, the rectification of the K_{ATP} channel is much weaker than with I_{K_1} (in that the conductance in the outward direction is larger) and is due to

Mg^{2+} block of the channels (Findlay 1987a, b, Trube & Hescheler 1984). The effects of intracellular polyamines are less marked than for strong rectifiers as the sites at which the polyamines bind at in TM1 and the C-terminal domain are neutral whereas in strong rectifiers they are negatively charged (Yamada and Kurachi, 1995).

ATP_i closes K_{ATP} channels in the heart with an IC_{50} of 20-30 μM and this is independent of whether the ATP is in the free acid form or Mg^{2+} bound (Findlay *et al.*, 1988; Lederer *et al.*, 1989; Terzic *et al.*, 1995). ATP_i increases the time between bursts of channel activity and decreases the duration of the

bursts with no change in the amplitude of the single channel current or in the kinetics of the open channel (Takei *et al.*, 1985; Spruce *et al.*, 1987). This role of ATP_i is referred to as the 'ligand action' as it is thought to require the binding of ATP to the channel.

Binding of ATP to Mg²⁺ does affect the potency of ATP in other cell types and in pancreatic β -cells the IC₅₀ value for Mg-ATP is 26 μ M compared with 4 μ M for the block by ATP. This indicates that unbound ATP (ATP⁴⁻) is more potent than Mg-ATP (Ashcroft *et al.*, 1991). In smooth muscle cells Mg-ATP is not effective in blocking the channels whereas ATP⁴⁻ blocks with an IC₅₀ of 29 μ M (Nelson *et al.*, 1995; Kajioka *et al.*, 1991).

The block by ATP_i is reduced in the presence of ADP and the IC₅₀ value for block by ATP increases almost fourfold in the presence of 100 μ M ADP (Findlay *et al.*, 1988; Lederer and Nichols 1990; Weiss *et al.*, 1990). ADP also increases P_o in the absence of ATP and this requires Mg²⁺ (Lederer and Nichols 1990; Weiss *et al.*, 1990). ATP is thought to bind at two sites at the channel and the binding at one increases the affinity of the second site for ATP. The binding of ATP to the second site is necessary for channel inhibition. Channel inhibition requires no Mg²⁺ whereas channel stimulation after rundown does and it has been suggested that MgADP and ADP³⁻ have separate binding sites (Findlay, 1988). In the absence of Mg²⁺, mono-, di- and triphosphates compete for the second site and inhibit the channel whereas in the presence of Mg²⁺, ADP binds to the first site without increasing the affinity of the second site for ATP (Lederer and Nichols 1989; see Nelson and Quayle, 1995 for a review).

K_{ATP} channel activity has been observed to decline after the excision of inside-out patches into ATP-free solution. This occurs in, for example, isolated patches of cardiac membrane where it is thought that the rundown is due to the decrease in ATP and a subsequent lack of phosphorylation, as non-hydrolyzable analogues of ATP do not stop run-down of the channel (Trube and Hescheler, 1984). The dependence of channel activity on phosphorylation has also been demonstrated in non-cardiac tissue, for example in urinary bladder and insulinoma cell lines where K_{ATP} channel activity is modulated by protein

kinase C (Wang and Giebisch 1991; Bonev *et al.*, 1993). The run-down seen after excision of membrane patches into ATP free solution is reversed by treatment with Mg-ATP (Ohno-Shosaku *et al.*, 1987, Takano *et al.*, 1990). Thus prevention of channel rundown requires Mg^{2+} -dependent hydrolysis of ATP and the effect lasts up to ten minutes after removal of ATPi. The need for Mg^{2+} may be due to kinase activity and the maintenance of channel activity may be due to an 'enzyme dependent' action of ATP (Terzic *et al.*, 1995).

Hence it can be concluded that the effect of ATPi depends upon the channel state. When the channels are open ATP closes them and when they close after run- down, treatment with Mg^{2+} -ATP restores channel activity.

Several drugs which could prove useful in the treatment of hypertension, acute and chronic myocardial ischaemia and congestive heart failure have the ability to open K_{ATP} channels. One such K^+ -channel opener (KCO) is cromakalim, which decreases the action potential duration (as does hypoxia) by opening K_{ATP} channels in cardiac muscle. The active enantiomer of cromakalim, levcromakalim, opens ventricular K_{ATP} channels hence decreasing the action potential duration and the amplitude and duration of evoked guinea-pig ventricular contraction (Sanguinetti *et al.*, 1988; Findlay *et al.*, 1989). The channels activated by levcromakalim were thought to be the same as those activated during dialysis of the cell or of patches with low concentrations of ATPi (Findlay *et al.*, 1989). Levcromakalim has been shown to open the K_{ATP} channel and inhibit the delayed rectifier current in the rat portal vein, and it was initially suggested that the K_{ATP} channel activated by levcromakalim is a voltage-insensitive state of the delayed rectifier (Ibbotson *et al.*, 1993; Edwards and Weston, 1993; Edwards *et al.*, 1995; Heath and Terrar, 1996a). However as already discussed in Section 1.2.2 molecular biology has now provided decisive structural evidence showing that the K_{ATP} channel and the delayed rectifier channels are quite different entities.

K_{ATP} channels have different sensitivities to the KCOs depending upon cell type. The pancreatic β -cell K_{ATP} can be activated by diazoxide and weakly activated by pinacidil. In contrast the cardiac K_{ATP} is activated by pinacidil but cannot be activated by diazoxide. The K_{ATP} in smooth muscle cells can be

activated by both of these KCOs (Ashcroft and Ashcroft, 1990; Nichols and Lederer, 1991; Terzic *et al.*, 1995; Nelson and Quayle, 1995). As the properties of the K_{ATP} channels vary between tissues, it may be that the channels are composed of heterogeneous subunits.

The conductance of the channels in cardiac myocytes is between 70 and 90pS in 140mM symmetrical K^+ (Noma 1983, Horie *et al.*, 1987, Ashcroft & Ashcroft 1990). The single channel conductance in other tissues varies. In rat portal vein two types of K_{ATP} channels have been identified (Zhang and Bolton, 1996). One was termed a LK (larger conductance) channel and had a similar conductance (50pS) and other properties to K_{ATP} channels described in other tissues. The second type was termed MK (smaller conductance) and has a lower conductance of 7-22pS. It resembles the type activated by intracellular NDPs (nucleoside diphosphates) and is thought to be the target of K^+ channel openers (Beech *et al.*, 1993). Two types of K_{ATP} channel have also been found in many smooth muscle cells. For example in portal vein (Beech *et al.*, 1993), cultured coronary artery cells (Miyoshi *et al.*, 1992) and urinary bladder (Bonev *et al.*, 1993) there is a small to intermediate conductance channel of between 15-50pS. Large conductance channels of ~130pS are found in the mesenteric arteries (Standen *et al.*, 1989), rat tail artery (Furspan and Webb, 1993) and canine aorta smooth muscle (Kovacs *et al.*, 1991).

The channel openings maintained by KCOs are affected by the NDPs and can occur spontaneously or after channel rundown (Terzic *et al.*, 1995; and Isomoto *et al.*, 1997). For example, pinacidil and levcromakalim are effective when applied during spontaneous channel activity and after rundown in the presence of NDPs. The mechanism of activation by KCOs is dependent on the metabolic conditions of the cell (Terzic *et al.*, 1995; and Isomoto *et al.*, 1997).

It has thus been proposed that during metabolic inhibition the NDP concentration rises and ATP_i falls which may result in the activation of the channel (Zhang and Bolton 1995). However, it has been noted that NDPs also regulate a K^+ channel whose activity is similar to the K_{ATP} channel in rabbit portal veins and rat mesenteric arteries (sometimes referred to as K_{NDP}) (Beech

et al., 1993a, 1993b) and this current is also blocked by ATP_i and glibenclamide with an IC₅₀ for glibenclamide of 25nM (Beech *et al.*, 1993a). It has been suggested that the application of K⁺ channel openers results in the activation of these channels (Beech *et al.*, 1993b, Zhang and Bolton, 1995). The situation in smooth muscle cells is thus not clear. The situation in cardiac tissue may be less complex, and this is discussed further in Chapter Five.

1.4.3.1: MOLECULAR BIOLOGY OF K_{ATP} CHANNELS.

As already discussed in Section 1.2.2, the β -cell K_{ATP} channel is composed of two subunits, a Kir channel subunit - Kir6.2 (BIR) - and a sulphonylurea receptor which is discussed in detail in the next section. The reconstituted complex was inwardly rectifying, sensitive to ATP_i, inhibited by sulphonylureas and activated by the KCO diazoxide. Kir6.2 (BIR) was screened from the human genomic library using Kir6.1(uK_{ATP}-1) as a probe. The amino acid sequence showed that Kir6.1 has 70% sequence homology with Kir6.2. It has 40-50% homology with other Kir channels. It has two transmembrane domains and a H5/P region motif which is Gly-Tyr-Gly in all other inward rectifiers but, interestingly, is Gly-Phe-Gly here.

The mRNA for Kir6.2(BIR) is strongly expressed in pancreatic β -cells, brain, skeletal muscle, cardiac muscle and insulinoma cells, and is weakly expressed in lung and kidney, but not found in the spleen, liver or testis (Sakura *et al.*, 1995). The second transmembrane domain had an asparagine at position 160 (analogous to aspartate at positions 171/172 in strong rectifiers) and there is a serine at position 212 so that the channel is weakly rectifying. The amino acids in analogous positions in strong rectifiers are negatively charged (discussed in detail earlier).

Kir6.1(uK_{ATP}-1) also couples to the SUR and displays sulphonylurea sensitivity when expressed with the SUR in HEK293 cells (Ammala *et al.*, 1996). The K_d for binding of glibenclamide was 6.8 ± 1.8 nM which is close to that for the native β -cell receptor of 0.3-7nM (Ashcroft and Ashcroft 1992). The SUR receptor does not itself act as an ion channel but provides the complex with sensitivity to sulphonylureas. In the HEK293 cells it was found to couple

to more than one type of Kir channel. However the coupling of the SUR to the endogenous Kir channels of the HEK293 cells did not result in ATP sensitivity - even though it does regulate the ATP sensitivity of the β -cell K_{ATP} . The presence of sulphonylurea sensitivity does not, therefore, necessarily indicate the presence of a K_{ATP} channel.

1.4.3.2: THE SULPHONYLUREA RECEPTOR

The sulphonylurea receptor is a member of the ATP Binding Cassette (ABC) superfamily and was first cloned from insulinoma cells (Aguilar-Bryan *et al.*, 1995). It has 13 transmembrane domains, 2 nucleotide binding folds with Walker A and B consensus motifs and an external NH_2 terminus. It also contains 2 N-linked glycosylation sites and 20 PKA- and 3 PKC- dependent phosphorylation sites. The mRNA for the receptor has been found in the pancreas, brain, heart and the kidney.

Co-expression of a mouse (m)-BIR and a hamster (ha)-SUR1 in *Xenopus* oocytes results in a K^+ conductance which is blocked by ATP with an IC_{50} of $10\mu M$ and by glibenclamide with an IC_{50} of $1.8nM$ and by tolbutamide with an IC_{50} of $32\mu M$. The channel is activated by diazoxide with an EC_{50} of $60\mu M$. These sensitivities to the blockers are similar to those of the β -cell K_{ATP} channels (Inagaki *et al.*, 1995).

The mRNA for the SUR1 has been detected in large amounts in the pancreatic β -cells and in small amounts in the brain (Inagaki *et al.*, 1995; Aguilar-Bryan *et al.*, 1995). An additional homologue - SUR2 - has been isolated from the rat brain (Inagaki *et al.*, 1996) and an isoform of SUR2 was cloned from a mouse heart cDNA library (Isomoto *et al.*, 1996). Another member was found to differ from this mouse SUR2 isoform by 42 amino acids in the C-terminal domain. The above mentioned SURs are now termed SUR1, SUR2A and SUR2B. The rat rSUR1 has 65-70% sequence homology with the mouse mSUR2A and mSUR2B.

The co-expression of Kir6.2 with either SUR2A or SUR2B results in a K_{ATP} channel with a single channel conductance of $\sim 80pS$. The co-expression of rSUR2A/mKir6.2 elicits channel activity which is inhibited by ATP_i with an

IC₅₀ of 100 μM and by 1 μM glibenclamide. The channel can be activated by pinacidil and cromakalim but not diazoxide (Inagaki *et al.*, 1996). These characteristics have also been observed in the cardiac and skeletal muscle K_{ATP} channels. (Fosset *et al.*, 1988; Faivre *et al.*, 1990; Nichols *et al.*, 1991; Terzic *et al.*, 1994). The mSUR2B/mKir6.2 complex has features similar to the K_{ATP} channel in smooth muscle cells (Beech *et al.*, 1993) such as activation by both pinacidil and cromakalim. The different SURs have different sensitivities to block by sulphonylureas. Tolbutamide is ~50 times less potent in the heart than in the β-cells and glibenclamide inhibits the channel in the heart and smooth muscle with an IC₅₀ of 5-20 μM compared with 4 -50 nM in β-cells (Zunkler *et al.*, 1988a, b, Ripoll *et al.*, 1990).

Distribution studies have shown that the mSUR2A is located in the atrium, ventricles, skeletal muscle, cerebellum, eye, and urinary bladder whereas mSUR2B is distributed not only in these tissues but also in the forebrain, liver, lungs, kidney, spleen, stomach, small intestine, colon, uterus, ovary, and fat tissue (Isomoto *et al.*, 1996). This is in keeping with the idea that these two SURs expressed with Kir6.x subunits constitute cardiac and smooth muscle K_{ATP} channels.

1.4.3.3: ATP-DEPENDENT KIR SUBFAMILY: THE PRESENCE OF A C-TERMINAL ATP-BINDING DOMAIN.

As already mentioned in Section 1.2.2 these Kir family members have a putative ATP-binding domain in their C-terminal domain. Kir1.1(ROMK1) is expressed predominantly in the kidney where it plays a part in electrolyte transport. It has been shown that the Kir1.1 protein is localized in the apical membrane of the cortical collecting duct in epithelial cells. Expression in oocytes results in a channel with a single channel conductance of 35-45 pS in 90 mM [K⁺]. Mg-ATP_i is needed for opening and the channel is inhibited by intracellular H⁺. These properties are similar to those of the channel found in the cortical collecting duct cells (Wang *et al.*, 1992; Ho *et al.*, 1993).

Kir4.1(K_{AB}-2) has 53% sequence homology with Kir1.1. Expression in oocytes leads to strong inwardly rectifying channels which are constitutively

active (Bond *et al.*, 1994) whereas Kir1.1 is a weak inward rectifier. The charge at two sites in the channel structure, R1 in the M2 domain and R2 in the C-terminal domain - Mg^{2+} and polyamine binding sites, as discussed in Section 1.3.3 and illustrated in Figure 1.3.3.1 - in Kir1.1 are both neutral. The charge at R2 in Kir4.1 is negative (Yang *et al.*, 1995). It is thought that Kir4.1 may associate with other Kir channel subunits to form heteromeric channels (see Isomoto *et al.*, 1997).

The mRNA for Kir4.1 is expressed in the kidney where it is found specifically in the basolateral membrane of distal tubular epithelial cells (Ito *et al.*, 1996). It may provide the K^+ for the Na^+-K^+ pump activity which underlies the net movement of univalent cations across these cells. In the brain Kir4.1 mRNA is expressed in the cerebellum, corpus callosum, hippocampus, thalamus, inferior colliculus and the brain stem. It is mainly found in the glial cells of the CNS and so may play a role in K^+ buffering action.

1.5: OTHER INWARDLY RECTIFYING POTASSIUM CHANNELS.

1.5.1: Na^+ -ACTIVATED CHANNELS.

These were first identified in cardiac tissue (Kameyama *et al.*, 1984). They are activated when intracellular Na^+ levels increase to above 20mM. They have the highest conductance (more than 200pS) of all the ion channels in the heart. Under normal physiological conditions the concentration of Na^+ inside the cell is about 10mM so these channels are thought to be active only during pathophysiological conditions when $[Na^+]_i$ increases significantly, for example, during ischaemia, digitalis intoxication and during perfusion with Ca^{2+} and Mg^{2+} -free medium. The channel is also present in neuronal tissue where it may play a role in repolarisation during the action potential following activation by the influx of Na^+ ions. A cDNA coding for the channel has not yet been isolated.

1.6: BLOCK OF K^+ CHANNELS BY CLOTRIMAZOLE , CETIEDIL AND THEIR ANALOGUES.

Very few compounds have been identified as selective blockers of inward rectifier channels. The compounds clotrimazole and cetiedil have recently been tested for this action in our laboratory as they are known blockers of other kinds of K^+ channel. Clotrimazole is an antifungal agent which is now known to block K_{Ca} channels in red blood cells (Alvarez *et al.*, 1992; Benton 1995).

Additionally the $I_{K(Ca)}$ current in murine erythroleukemia cells and in PC12 cells and the whole cell K^+ current in ferret portal vein smooth muscle cells are blocked by clotrimazole at concentrations of 10nM to 10 μ M (Rittenhouse *et al.*, 1994a, 1997). Clotrimazole is thought to block the K^+ channel directly, independent of its inhibitory action on the cytochrome p450 epoxygenase (Alvarez *et al.*, 1992; Rittenhouse *et al.*, 1994b, Vandorpe *et al.*, 1997). Other recent studies have shown the block of K^+ currents in many tissue types by clotrimazole (Couprie *et al.*, 1996; Brugnara *et al.*, 1993; 1995; 1996).

Cetiedil is an antisickling agent and can block K_{Ca} channels in red blood cells (Christophersen and Vestergaard-Bogind, 1985; Benton *et al.*, 1994), volume-activated K^+ channels in rat hepatocytes (Sandford *et al.*, 1992) and levcromakalim-activated K^+ channels in smooth and skeletal muscle (Benton 1995). A previous study in rat atrial myocytes by Dr. A.G. Jones in our laboratory has shown that the G-protein gated K^+ channel can be blocked by clotrimazole and cetiedil, as well as by some of their structural analogues. The block by clotrimazole was observed to be use-dependent. Moreover, an analogue of cetiedil, UCL1495, was found to be very potent with an IC_{50} of 0.4 μ M. (Jones, 1995). This compound has been shown to be much more effective than cetiedil as an $I_{K_{Ca}}$ blocker in rabbit erythrocytes (Benton *et al.*, 1994) whereas it had little effect on levcromaklim-induced current in rat aorta (Benton, 1996).

1.7: BARIUM BLOCK OF INWARD RECTIFIERS:

Ba^{2+} is a well characterised blocker of K^+ channels. It blocks delayed rectifiers from the outside, $K_{(Ca)}$ from the inside, and the background inward rectifier and K_{ATP} from the outside (Hille 1992). The block of the channel

occurs with an IC_{50} of $2.2\mu M$ at $-120mV$ in pulmonary artery endothelial cells (Voets *et al.*, 1997), $12\mu M$ at $-65mV$ in skeletal muscle (Standen and Stanfield, 1978) and $1.3\mu M$ at $-120mV$ in Purkinje fibres (Carmeliet and Mubagwa, 1986). The block by Ba^{2+} has been reported to be time and voltage-dependent in the I_{K_1} of the SA node (Osterrieder *et al.*, 1982), I_{K_1} in atrial cells (Bechem *et al.*, 1983) and in Purkinje fibres (DiFrancesco *et al.*, 1984) as well as in non-cardiac tissue (Armstrong and Taylor 1980, Hagiwara *et al.*, 1978, Standen and Stanfield 1978). The block by external Ba^{2+} is more marked at negative than at positive potentials. The voltage-dependence indicates that Ba^{2+} penetrates the cell membrane. The depth of the binding site within the membrane can be indicated by μ [which is the fraction of the total electrical potential drop between the outside of the channel and the binding site in the channel, and is often referred to as the 'electrical distance' (Hille, 1992)] which has a value between 0 and 1 (Hagiwara *et al.*, 1978). The value for I_{K_1} is 0.64 - 0.68 in starfish egg and 0.64 - 0.74 in skeletal muscle fibres indicating that the binding site is close to the inner mouth of the channel.

IC_{50} values for block of $I_{K_{ACh}}$ by Ba^{2+} have been estimated as $125\mu M$ at $0mV$ and $15.3\mu M$ at $-80mV$ in guinea-pig atrial cells (Zang *et al.*, 1995); $11\mu M$ at $-70mV$ in rabbit Purkinje fibres (Carmeliet and Mubagwa, 1986). The block of $I_{K_{ACh}}$ is described by 1:1 binding of Ba^{2+} to a site within the channel. The value of μ for $I_{K_{ACh}}$ in guinea-pig atrial cells is ~ 0.3 which indicates that the binding site is near the extracellular face of the membrane (Zang *et al.*, 1995). Ba^{2+} is considered to cause an open channel block, as its action is dependent upon the extracellular K^+ concentration and is also time- and voltage-dependent. The initial peak of current induced by adenosine is not significantly reduced by the pre-application of Ba^{2+} , indicating that there is no block of closed channels (Zang *et al.*, 1995).

Block by Ba^{2+} of K_{ATP} channels is voltage-dependent in frog skeletal muscle with an IC_{50} of $0.1mM$ at $-62mV$ (Quayle *et al.*, 1988). In mouse pancreatic β -cells the block is also voltage-dependent and the IC_{50} value is $0.18mM$ at $-62mV$ and $12.5\mu M$ at $-123mV$ (Takano and Ashcroft 1996). The voltage-dependence is attributed to Ba^{2+} binding at a site within the channel so

impeding the flow of K^+ ions. Single channel studies by Quayle *et al.*, (1988) have shown that Ba^{2+} decreases the mean channel lifetime compatible with an open channel block. However, Takano and Ashcroft (1996) found that K^+ ions had no effect and proposed that Ba^{2+} binds to a site outside the pore, inducing closure of the channel. They also argued that the study in skeletal muscle did not fully examine the effects of increasing external K^+ on the Ba^{2+} block. The explanation for these differences remains unclear.

To summarise, it appears that Ba^{2+} is most effective on the I_{K1} channel followed by $I_{K_{ACh}}$ and finally I_{ATP} . The block of I_{K1} and $I_{K_{ACh}}$ is voltage dependent and is thought to be due to open channel block whereas the nature of the block of I_{ATP} remains unclear. The different potencies may be attributed to the amino acid residues present at important sites within the channel structure as discussed previously in Section 1.3.3. The residues at R1 and R2 in I_{K1} are negative as is R1 in $I_{K_{ACh}}$. As these are important in binding of Mg^{2+} and polyamines, it may be that there are some residues present which are important in the binding of Ba^{2+} . The corresponding residues in K_{ATP} are neutral which may decrease the affinity for Ba^{2+} and possibly reduce the voltage-dependence of the blocking action. As of yet no one has thought to correlate Mg^{2+} /polyamine block or degree of rectification with sensitivity to Ba^{2+} , though a relationship seems possible.

1.8: OTHER BLOCKERS OF INWARD RECTIFIERS:

The I_{K1} channel is blocked from the outer side of the membrane by TEA, Cs^+ , Rb^+ , Na^+ and Sr^{2+} and by H^+ from the inside (Hille 1991). I_{K1} in skeletal muscle is blocked by TEA with an IC_{50} of 20mM (Stanfield, 1983).

K_{ATP} channels in pancreatic β -cells are blocked by 25% by 10mM TEA (Cook and Hales, 1984) and in ventricular cells 1mM causes an almost complete block (Takei *et al.*, 1985). 4-AP at 1mM abolishes K_{ATP} channel openings in guinea-pig ventricular cells (Takei *et al.*, 1985) and in pancreatic β -cells the channel is blocked by 10mM (Cook and Hales, 1984). However the channel activated by metabolic exhaustion of frog skeletal muscle is insensitive to



similar concentrations of 4-AP (Castle and Haylett, 1987). The ATP-current is also blocked by glibenclamide, tolbutamide, phentolamine, ciclazindol and lidocaine (Edwards and Weston, 1993). Blockers of the atrial $I_{K_{ACh}}$ include quinidine, having an IC_{50} of $\sim 10\mu M$ (Nakajima *et al.*, 1989), disopyramide ($IC_{50} = 1.7\mu M$) and flecainide ($IC_{50} = 3.6\mu M$) (Inomata *et al.*, 1991). The channel is also blocked by Cs^{2+} , 4-AP, TEA and quinine.

1.9: AIMS OF PRESENT STUDY:

The primary aim of the present work was to examine the effects of compounds structurally related to drugs tested previously as potential inhibitors of $I_{K_{ACh}}$ in neonatal rat cardiac tissue. These compounds were also tested on I_{K_1} and I_{ATP} in order to determine their possible selectivity. Selectivity would not be unexpected in view of the structural differences between the three channels, as is the case for the rectification due to intracellular block by Mg^{2+} and polyamines, as discussed in Section 1.3.3.

Ba^{2+} was also used as a well characterised inhibitor displaying an open-channel block and compared with published results in order to determine its selectivity between the three channels in the neonatal rat heart. The block by Ba^{2+} is also affected by residues in the channel structure (see Section 1.7 and Chapter Six) and it was hoped the findings with Ba^{2+} might be useful in interpreting the effects of other inhibitors, including the new compounds to be tested.

The effect of glibenclamide on $I_{K_{ACh}}$ and I_{ATP} was also studied in some detail in the hope that this would provide information as to whether or not the current induced by application of adenosine in atrial myocytes included a component of I_{ATP} as has been suggested by Li *et al.*, (1995). These authors observed that the current activated by adenosine in guinea pig atrial cells was susceptible to inhibition by glibenclamide. As glibenclamide was considered to be a specific blocker of K_{ATP} channels, it was concluded that the application of adenosine resulted in the activation of not only G-protein gated channels but also K_{ATP} channels. It was also hoped that the present examination of the effect of glibenclamide on all three channels would be useful in determining whether

the SUR associated with inward rectifier channel subunits other than K_{ATP} subunits, as has already been described by others (see Section 1.4.3.1.).

CHAPTER TWO : METHODS

CHAPTER TWO: METHODS.

2.1: TISSUE CULTURE

2.1.1: PREPARATION OF NEONATAL RAT CARDIAC MYOCYTES

A previous procedure developed in this laboratory, and described by Jones (1995) was modified for use in the preparation of both atrial and ventricular myocytes. A one day old rat pup (of either sex) was killed by cervical dislocation and the thorax opened. The heart was removed into Hanks balanced saline solution in a Falcon culture dish. This solution was supplemented with Ca^{2+} , Mg^{2+} and EGTA (see below) as this made cells more robust and calcium-tolerant. The Hanks solution used throughout the isolation procedure had been pre-heated to 37 °C.

The atria and ventricular tissues were separated using a dissecting microscope and transferred into fresh solution in separate dishes. The atria were ‘nicked’ with scissors to increase the surface area for enzyme access and ventricles were cut into $\sim 1\text{mm}^3$ pieces. After dissection the solution was replaced with one containing 2 mg / ml collagenase and 6 mg / ml bovine serum albumin. The tissue was then incubated in this solution at 37 °C for 35 minutes. It was next place in Hanks containing 2.5 mg / ml trypsin at 37 °C for a further 10 minutes. A final incubation in this solution, but now enzyme free and containing 6 mg / ml bovine serum albumin, at room temperature for 10 minutes served to arrest further enzyme activity.

Cells for electrical recording were isolated by dabbing pieces of tissue to the bottom of a culture dish containing Hanks solution. Single cells detached from the tissue and stuck readily to the base of the dish. The tissue was not triturated at any time. Cells were left in this solution for 2 hours before use and some were incubated in culture medium (see below) at 37 °C (95% O_2 + 5% CO_2) for use the next day.

The composition of the isolation medium and of the culture medium are given in Section 2.1.2.

The two different cell types did not appear vastly different. Atrial cells were 7-10 μm in diameter and appeared elongated and striated. After 2 hours in culture dishes, they became rounded. Ventricular cells were slightly bigger with a diameter of 10-14 μm . Cells were less elongated but still striated. These cells retained their structure after 2 hours in culture. The next day both cell types had become more spherical, the atrial ones more so than those from the ventricles.

2.1.2: ISOLATION AND CULTURE MEDIUM

Isolation medium was Hanks balanced salt solution without calcium or magnesium, from Gibco. This was supplemented with the following:

Ca^{2+} 20 μM

Mg^{2+} 1 mM

EGTA 100 μM

The free Ca^{2+} concentration was estimated to be 20 nM.

Cells were kept overnight in culture medium of the following composition:

L-15 (Leibovitz) medium with L-glutamine (42.6 ml)

Penicillin-Streptomycin (1 ml containing 200 IU-200 μg)

Foetal Calf Serum (5 ml)

24.9 mM Sodium bicarbonate (1.4 ml of 7.5%)

2.2: RECORDING TECHNIQUES.

2.2.1: MEDIUM.

During an experiment cells were constantly perfused with a physiological solution that was buffered with Hepes. The solution composition was as follows:

NaCl 136 mM

KCl 4.7 mM

CaCl₂ 1.0 mM

MgCl₂ 1.2 mM

N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes) 10 mM

Glucose 10 mM

The pH was adjusted to 7.4 with 1M NaOH.

This solution flowed under gravity into the 35mm diameter culture dish via an inlet tube made of stainless steel. The inlet was positioned to direct the inflowing solution directly onto the cell. The solution level was kept constant by a similar tube which was attached to a suction pump thus maintaining a constant volume in the recording chamber. The solution flowed at ~ 5 ml / min and was pre-heated by passage through a water jacket to give a temperature in the culture dish of ~ 31°C. Drugs were dissolved in the perfusion solution and stored in separate reservoirs. The perfusion could be switched from drug-free solution to one of 4 drug solutions. The time taken for a new solution to reach the cell was ~ 5s.

2.3: WHOLE CELL RECORDING TECHNIQUE.

2.3.1: PATCH PIPETTES.

Patch pipettes were pulled from thin walled borosilicate glass tubing (GC-150TF Clark Electromedical Instruments) containing an inner filament.

This was usually done using a List patch electrode puller (L/M-3P-A; List Medical), although in some cases an in-house horizontal puller was employed. Pipettes were not fire-polished and had resistance of between 8-14 M Ω . However, for experiments with GTP- γ -S, pipettes with resistance of between 3-6 M Ω were employed. Prior to use pipettes were backfilled with internal solution with the aid of a 1 ml syringe fitted with a 0.22 μ m Millipore filter.

2.3.2: INTERNAL SOLUTION

The internal solution was of the following composition:

K-Citrate ($K_3 C_6 H_5 O_7 \cdot H_2O$)	56mM
KCl	25 mM
NaCl	10 mM
MgCl ₂	1 mM
HEPES	35 mM
K-EGTA	1 mM
CaCl ₂	1.01 mM

This gave a free Ca²⁺ concentration of 49 μ M, and a free Mg²⁺ of 74 μ M as calculated using REACT (G.L. Smith, Department of Physiology, University of Glasgow, U.K.).

The pH of the solution was adjusted to 7.2 with 1 M KOH.

100 μ M GTP (Na-salt, Sigma), 3 mM ATP (Na-salt, Sigma) and 10 mM Mg²⁺ (MgCl₂) were added when activating the G-protein gated current via adenosine receptors. When it was desired to activate the channel directly through the G-protein, GTP was replaced with GTP- γ -S (Na-salt, Sigma). Internal ATP was left out when examining the levcromakalim-induced current in ventricular

myocytes (see Chapter Five).

2.3.3: VOLTAGE-CLAMP TECHNIQUES.

The culture dishes were placed in a hollow in a perspex block which was mounted onto the stage of an inverted Nikon TMS phase contrast microscope. This in turn was mounted on steel and concrete slabs supported on a Micro-g vibration isolation table (Technical Manufacturing Corporation) to minimise any external vibration. A Narishige hydraulic micro-manipulator system controlled the movement of the pipette. Screening from electrical interference was provided by a Faraday cage.

Whole-cell voltage-clamp recordings were carried out using a patch-clamp amplifier made in the Pharmacology Department UCL, by Mr Ted Dyett. PCLAMP 6 was used to generate command voltages and to record data. Voltage and current outputs from the patch amplifier were amplified five-fold, low-pass filtered at 1.5kHz (using an in-house 8 pole Bessel low pass filter) and digitised (Instrutech VR-10 digital data recorder). The pipette currents were displayed on the computer monitor, on a digital oscilloscope (Gould 20 MHz digital storage Type 1424), and on a pen recorder (Gould Easy Graf TA 240,). Data were stored on the hard drive of a PC (Acer) using the Axon TL1 Labmaster digitiser and the pCLAMP6 acquisition program and also on tape (Panasonic videocassette recorder NV-L20 HQ). The stored data were analysed with the aid of the pCLAMP 6 data analysis program Clampfit.

The patch pipettes were held in a Perspex holder (Clark Electromedical) which was attached directly to the head-stage of the patch amplifier mounted on the micro manipulator. The pipette and seal resistance were estimated by constant voltage pulses (usually -10mV lasting 10ms) by the 'test-seal' program provided by pCLAMP 6. The pipette was lowered toward a cell until a decrease in the current deflection indicated that the pipette was touching the cell

surface. Gentle suction was then applied and a large increase in resistance indicated the formation of a seal. The fast capacity transients due to pipette capacitance were then compensated. Once the seal resistance had reached at least 1 G Ω the holding potential was set to -50mV. A further application of suction ruptured the cell membrane underneath the pipette tip, and this was indicated by an increase in the capacity transients and in the current pulse. The transients due to cell membrane capacitance were compensated for before conducting the experiment proper, and the values of the membrane resistance and capacitance were noted.

The differences between the ionic compositions of the bathing solution and the cell are likely to lead to a change in the liquid junction potential after formation of the whole cell configuration. Junction potentials were estimated using the computer program JPCALC (Barry, 1994). This requires the total concentrations of internal and external ions to be known. However, citrate was not an option within the program. Selecting aspartate as a substitute gave a theoretical value of +9.4mV, and using acetate gave a value of +7mV. Using these values as guides it is estimated that the junction potential is \sim +10mV in the whole cell configuration. Thus in the current-voltage relationships presented in this study the reversal potentials are actually 10mV positive to their true value. Whilst this has been taken into account when interpreting the results obtained, corrections have not been applied to the 'raw' data presented.

2.4: DATA ANALYSIS.

Results presented are means \pm standard errors, and the number of observations are given either in parenthesis or in the figure legends. t-tests were used in order to assess statistical significance. Differences were considered significant when P was less than 0.05. Further details are given in the text. Log concentration-response curves were constructed by fitting the unweighted data to the Hill equation shown below using the method of least squares

minimisation. This was done using either Origin 4.1 (Microcal software) or CVFIT (written by Professor D. Colquhoun). This gave estimates for the IC₅₀ values and the Hill slopes.

$$Y = Y_{\max} \cdot \frac{[B]^n}{IC_{50}^n + [B]^n} \quad (\text{Equation 2.4})$$

2.4)

where

y_{\max} = maximum response

[B] = concentration of the blocker, B.

n = Hill coefficient.

IC₅₀ = half maximum inhibitory concentration of B.

The maximum responses were normally constrained to 100% since the agents tested were capable of completely abolishing the measured current.

CHAPTER THREE:
INHIBITORS OF THE G-PROTEIN-GATED K^+
CURRENT IN RAT ATRIAL MYOCYTES.

CHAPTER THREE: INHIBITORS OF THE G-PROTEIN-GATED K^+ CURRENT IN RAT ATRIAL MYOCYTES.

3.1: INTRODUCTION.

The main aim of the present study was to investigate the pharmacology of the G-protein-gated K^+ channel in atrial myocytes and to compare it with that of two other cardiac inward rectifiers, the background inward rectifier, I_{K1} , and the ATP-sensitive channel I_{KATP} . This chapter presents results from experiments on the G-protein-gated current.

Some compounds with known blocking actions on K^+ channels were examined first. Several of these, including clotrimazole and cetiedil, have been tested previously in rat atrial myocytes and other tissues in our laboratory (Jones, 1995). Novel compounds based on clotrimazole and cetiedil have since been developed in the Department of Chemistry at University College London and in the present work these have been tested extensively on this channel. They include a new analogue of clotrimazole, UCL1880, and an analogue of cetiedil, UCL1495, which has been previously tested in a preliminary fashion by Jones (1995).

The G-protein-gated K^+ channel in atrial cells can be activated by either adenosine or acetylcholine (Belardinelli & Isenberg, 1983, West & Belardinelli 1985, Kurachi *et al* 1987). In this study adenosine was employed since earlier work (Jones, 1995) had suggested that some of the compounds previously tested may block muscarinic receptors. However, a possible difficulty with the use of adenosine was that it has been reported that the actions of adenosine on atrial cells may include an activation of I_{KATP} . Li *et al* (1995) have shown that the sulphonylurea glibenclamide- generally taken to be highly selective in blocking K_{ATP} - is capable of blocking the adenosine response in guinea-pig atrial cells but not the response to acetylcholine. Also, Wang and Lipsius (1995) showed that acetylcholine activated a glibenclamide-sensitive K^+ channel in cat atrial myocytes suggesting that acetylcholine too activates the K_{ATP} channels. Li *et al.*, (1995) accordingly proposed that the current activated by adenosine in rat atrial myocytes may involve not only the G-protein-gated channel but also

activation of the K_{ATP} channels. An alternative explanation is that glibenclamide is not as selective for the K_{ATP} channels as has been thought. In order to address this issue glibenclamide was tested in the present work against the adenosine response. The concentration range over which glibenclamide blocked the action of adenosine was compared with that for its effect on the K_{ATP} channel in ventricular myocytes and with published values for the glibenclamide effect on K_{ATP} channels in other studies.

3.2: AIMS:

The overall aim was to establish the potencies of UCL1880, UCL1495 and glibenclamide in blocking the adenosine-induced current in rat atrial myocytes. The results were to be compared with the effects of the same compounds on two other cardiac inward rectifiers, I_{K1} and $I_{K_{ATP}}$. The effect of Ba^{2+} on the adenosine-induced current was also investigated in these cells.

3.3: RESULTS.

3.3.1: THE ADENOSINE RESPONSE

Figure 3.3.1.1A shows the effect of $1\mu M$ bath applied adenosine applied for approximately 30 seconds on an atrial myocyte clamped at $-50mV$. An initial outward current developed which reached a peak within 10 seconds and then decayed towards a new lower steady state level. The current falls to between 60-70% of the peak within the time of application of adenosine. At $-50mV$ adenosine activated a peak outward current of $235 \pm 40pA$ and mean steady state outward current (measured 25 seconds after initiation of the response) of $162 \pm 23pA$.

Figure 3.3.1.2A shows the current-voltage relationship of the control and adenosine-induced current from an atrial myocyte. The current activated by adenosine is inwardly rectifying and reverses at $-90mV$ which is close to the calculated E_K set for these cells ($-94mV$).

The whole cell control adenosine responses shown in Figure 3.3.1.1, 3.3.1.3 and 3.3.1.4 exhibit a decay in the outward current with time. This decay,

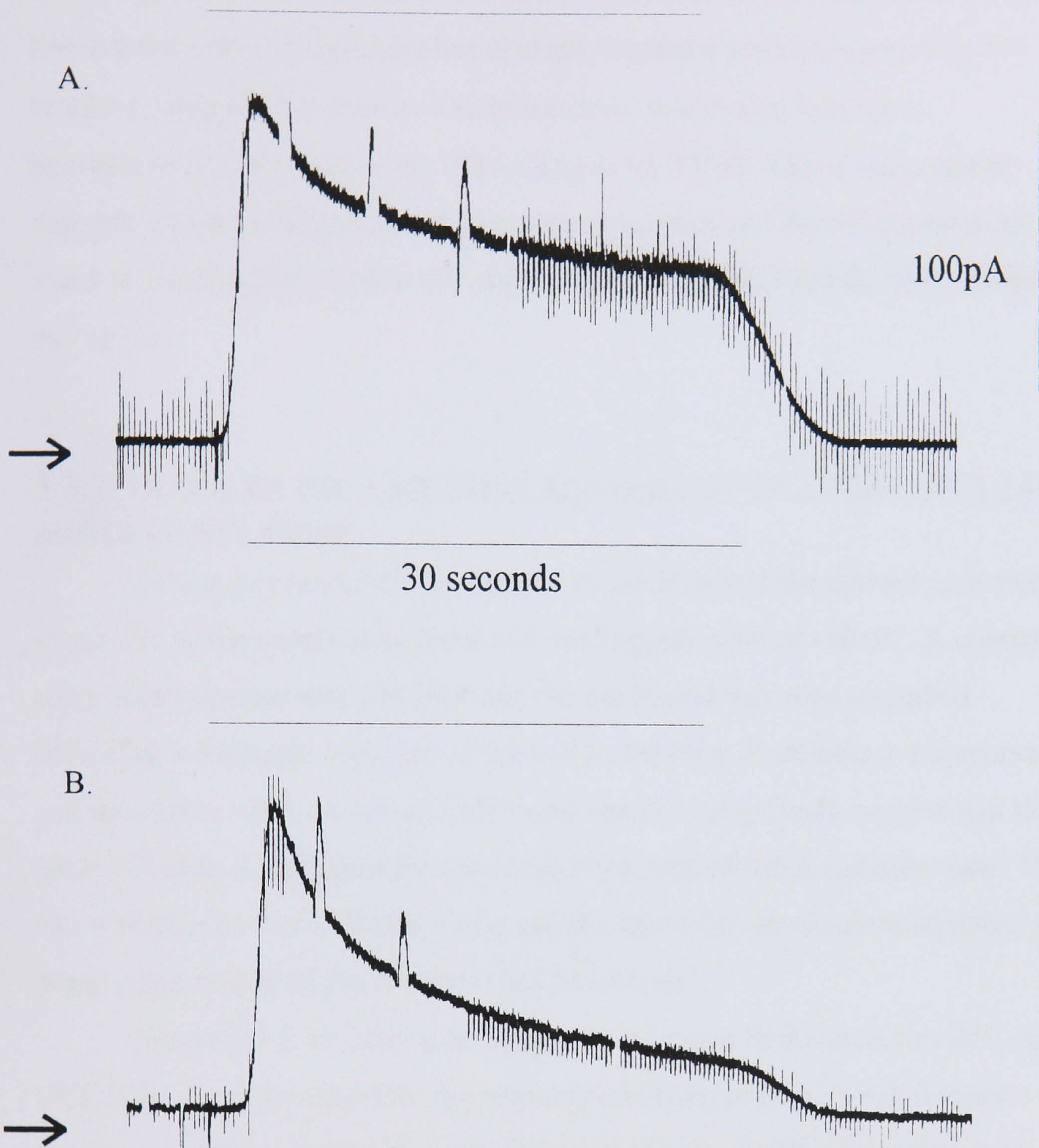


Figure 3.3.1.1. The effect of 0.2 μ M UCL1880 on the current activated by 1 μ M adenosine. A. Control response ; adenosine was bath applied for approximately 30 seconds and evoked an outward current. The membrane potential of the atrial cell was held at -50mV. B. Response of the same cell in the presence of 0.2 μ M UCL1880. The compound was applied 4 minutes prior to the adenosine application. The horizontal bar above each trace indicates the application time of adenosine. There is a delay before the response due to the time required for the new solution to reach the cell. The vertical deflections throughout the response are due to -10mV voltage steps. The larger deflections are in response to short voltage ramps but are not faithfully reproduced by the recorder. The arrows on the left of the traces indicate the zero current level.

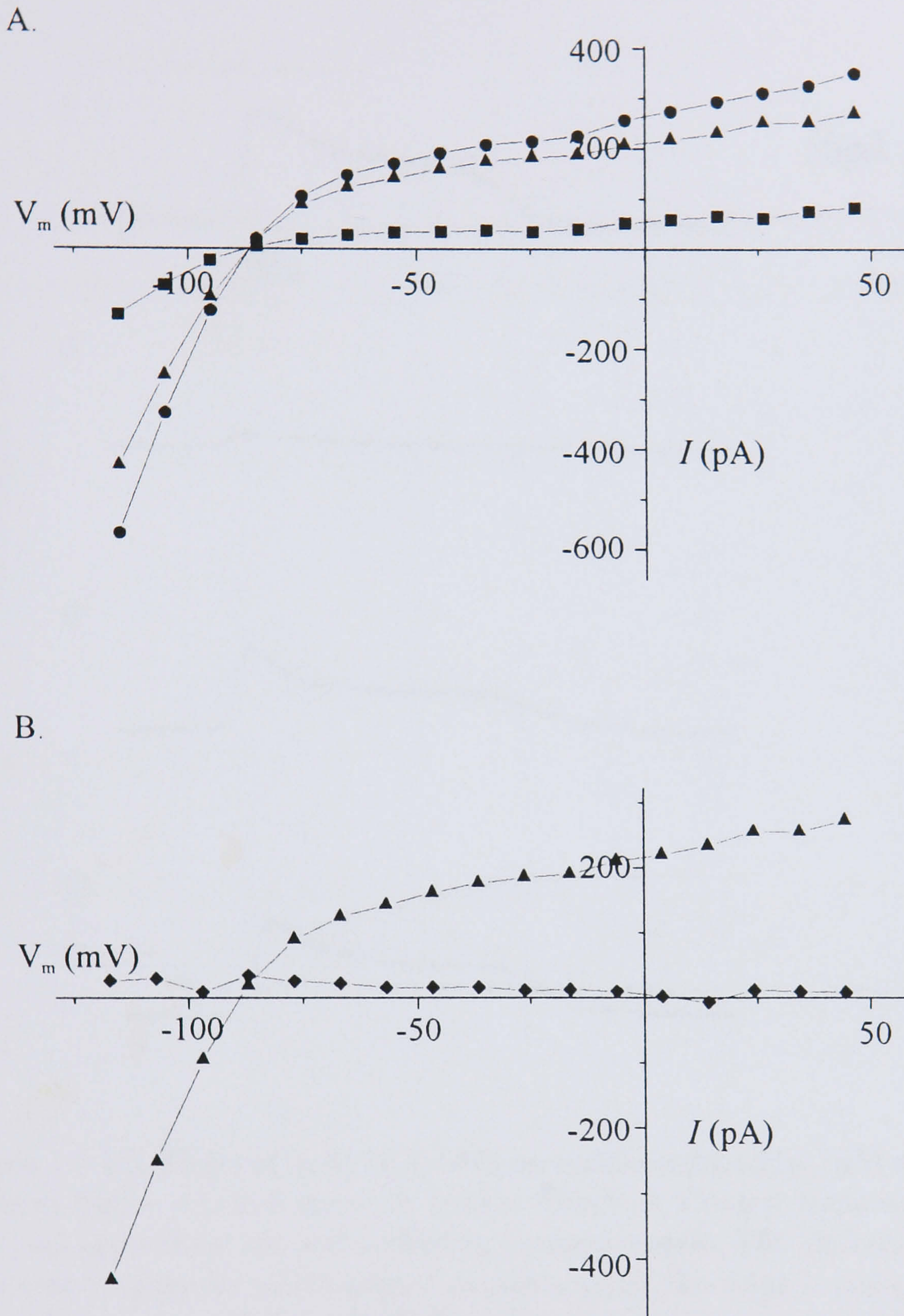


Figure 3.3.1.2. The steady state whole-cell current-voltage (I-V) relationship and the response to $1\mu\text{M}$ adenosine in rat atrial myocytes. A. The current was measured in control conditions (\blacksquare), in the presence of adenosine (\bullet), and subtracted to give the current due to adenosine (\blacktriangle). Each voltage step lasted 1 second and currents were measured near the end of the steps. B. The currents elicited by adenosine alone (\blacktriangle) and by adenosine in the presence of $2\mu\text{M}$ UCL1880 (\blacklozenge). Background currents were subtracted. Cells were held at a potential of approximately -50mV . The membrane potential was stepped from between -120 and $+40\text{mV}$ in $+10\text{mV}$ increments relative to the holding potential.

which has been analyzed in Section 3.3.2, varied from cell to cell and can be attributed to different amounts of desensitization. In atrial cells this is thought to involve not only the receptors but also subsequent events as, by-passing the receptor using GTP- γ -S gives rise to a current that is also subject to desensitization (Kurachi *et al*, 1987, Zang *et al*, 1993). This component of desensitization is thought to involve a phosphorylation / dephosphorylation event at the G-protein and/or the channel protein (Kim, 1991 & 1993; Zang *et al*, 1993).

3.3.2: BLOCK OF THE ADENOSINE RESPONSE BY UCL1880, UCL1495 AND GLIBENCLAMIDE.

Selected compounds were tested as inhibitors of the current activated by adenosine at the steady state level at a holding potential of -50mV. A control adenosine response was obtained and the adenosine was then reapplied following a 4 minute exposure of the cell to the drug. Preliminary experiments and the earlier work of Jones (1995) with similar compounds suggest that this period of time is sufficient for any blocking action to reach a steady state. The cell was then bathed in Krebs alone and the adenosine application repeated at 5 minute intervals until the response had recovered.

Figure 3.3.1.1B shows an adenosine response in the presence of 0.2 μ M UCL1880. It can be seen that the response has been greatly reduced at the steady state level whereas the peak response is only slightly diminished. At higher concentrations of UCL1880 the peak was reduced to a greater extent (see also the results with UCL1495, below). The current-voltage relationships for the steady state adenosine response alone and in the presence of a relatively high concentration of UCL1880 (2 μ M) are shown in Figure 3.3.1.2B.

Figure 3.3.1.3 shows the response of a different cell to adenosine alone and to adenosine in the presence of the cetiedil analogue UCL1495. The control response shows the characteristic peak and decline to a steady level. In the presence of 2 μ M UCL1495 both the peak and in particular the steady level are greatly reduced.

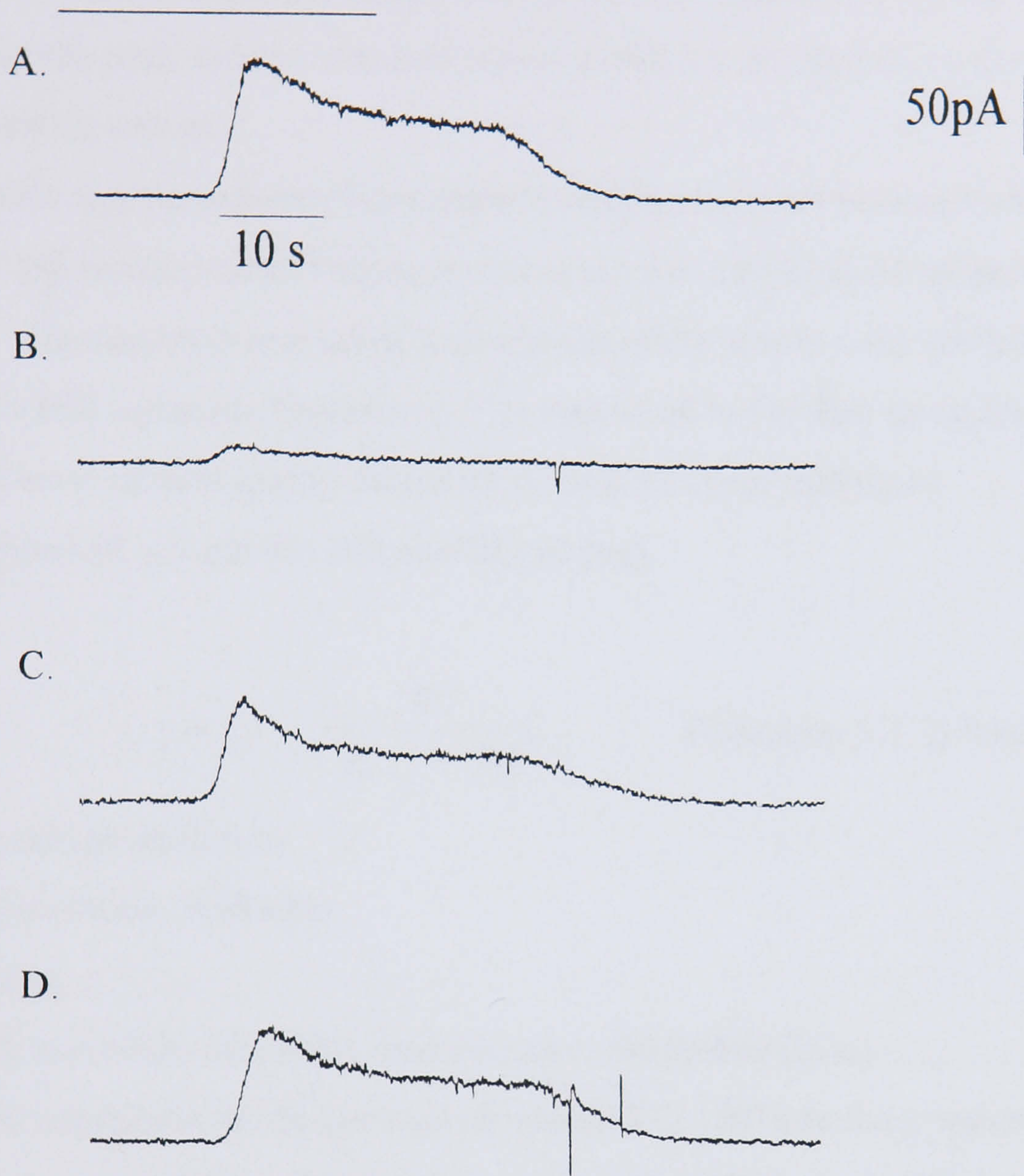


Figure 3.3.1.3. Effect of 2 μ M UCL1495 on current induced by 1 μ M adenosine. Records from a rat atrial myocyte held at -50mV. A. Control response; adenosine was bath applied for 30s and evoked an outward current. The horizontal bar indicates the time for which adenosine was applied; the delay is due to the time required for it to reach the cell. B. The adenosine response was greatly reduced in the presence of 2 μ M UCL1495, which had been applied 4 minutes beforehand. C and D. Recovery of the response at 5 and 10 minutes, respectively, after washout of UCL1495.

A different pattern of inhibition was seen when the ability of glibenclamide to block the response to adenosine was examined. Figure 3.3.1.4 illustrates the effect of glibenclamide at 20μM on the adenosine current. It can be seen that the peak and the steady state responses have both been reduced, and to a comparable extent.

Following these preliminary experiments a range of concentrations were tested and log concentration-response curves were constructed for all three inhibitors. The response was taken as the block of the steady state current at -50mV. The Hill equation (Equation 3.3.2) was fitted to the data using the method of least squares giving estimates of half-maximal inhibitory concentrations (IC₅₀) and the Hill coefficient (*n*_H).

$$Y = Y_{\max} \cdot \frac{[B]^n}{IC_{50}^n + [B]^n} \quad \text{(Equation 3.3.2) where}$$

*y*_{max} = maximum inhibition

[B] = concentration of blocker

n = Hill slope

IC₅₀ = half maximum inhibitory concentration of blocker (IC₅₀).

The maximum inhibition was constrained to 100% as there was no indication that any of these drugs were capable of producing more than 100% block. (High concentrations of inhibitor did not reveal an inward current in response to adenosine).

Figure 3.3.2 shows the log concentration-response curves for the effect of UCL1880, UCL1495 and glibenclamide on the adenosine response. It can be seen that all three compounds tested are active in the micromolar range.

Table 3.3.2 shows the IC₅₀ values and the Hill coefficients obtained from the curves for each compound.

These results indicate that UCL1880 with an IC₅₀ of 0.2μM is the most effective of the three in blocking the adenosine-induced current. It is also more active than the parent compound clotrimazole which has an IC₅₀ of 0.9μM (Jones 1995).

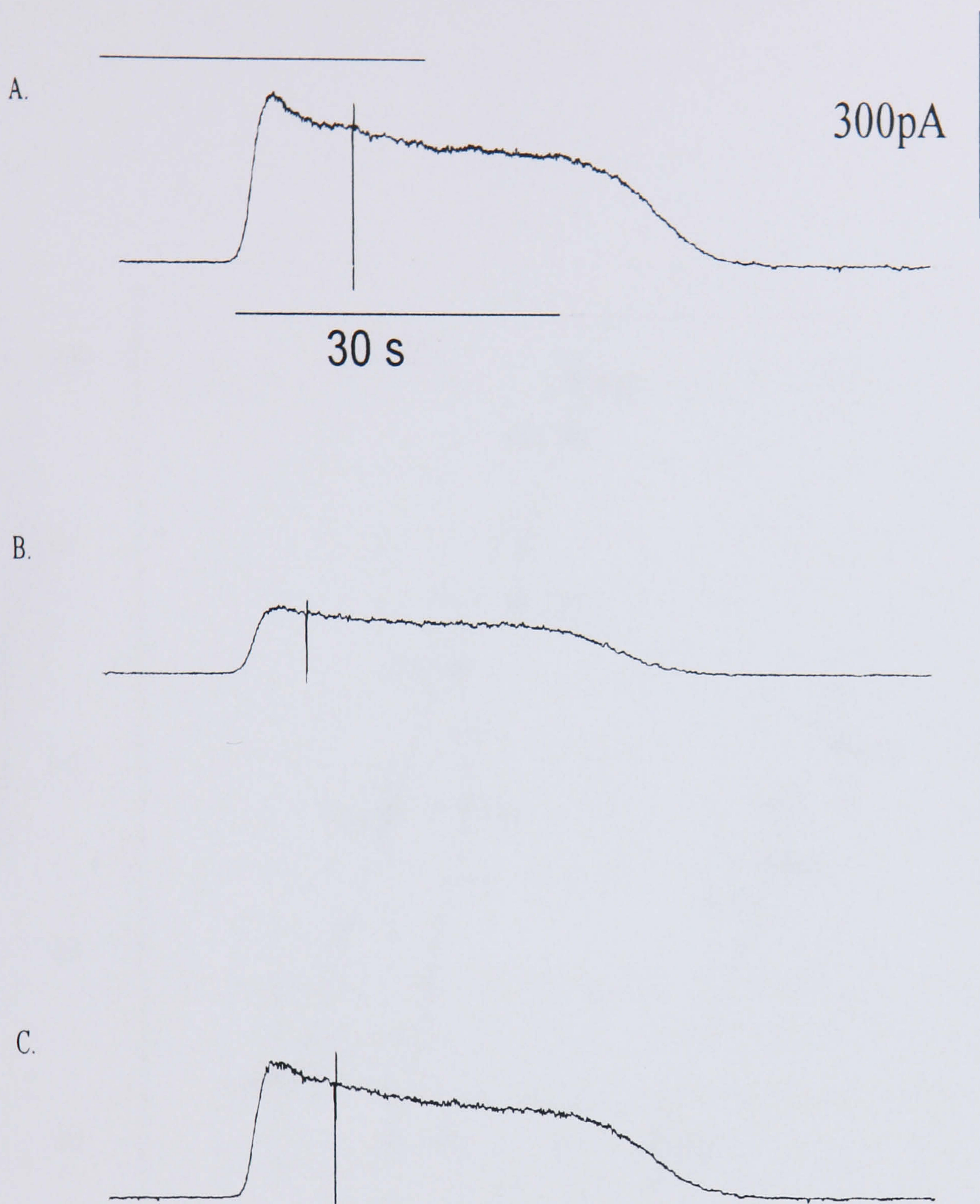


Figure 3.3.1.4: Effect of glibenclamide on current induced by 1 μ M adenosine. Records from a rat atrial myocyte cell held at -50 mV. (A), Control response; adenosine was applied for 30 seconds and evoked an outward current which declined to a lower level. The horizontal bar indicated the time of adenosine application; delay is due to time required to reach the cell. (B), Preincubation of the cells in 20 μ M glibenclamide for 4 minutes decreased the response with little change in its overall shape. (C), Recovery of response after 5 minute washout with Krebs solution.

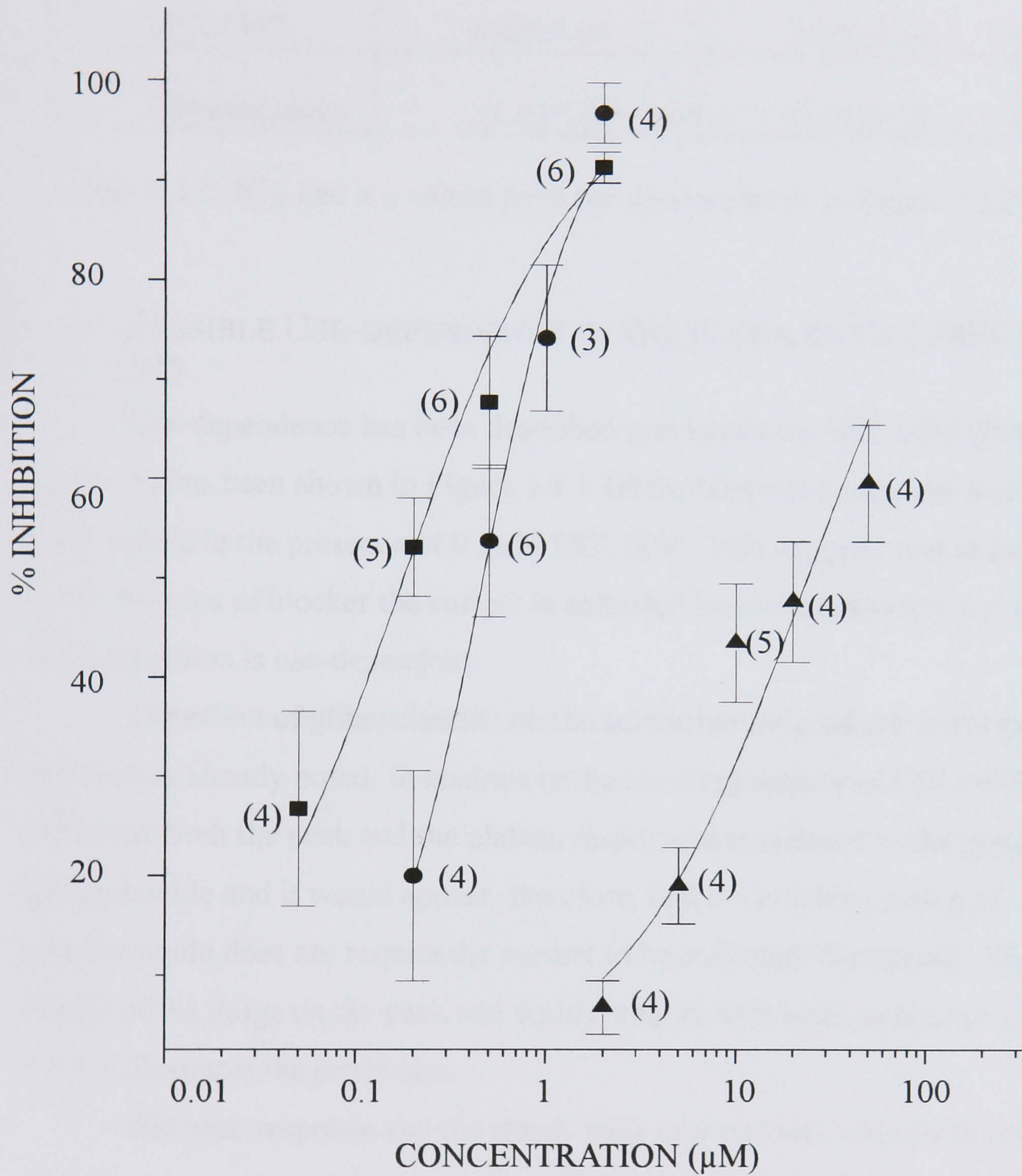


Figure 3.3.2: Log-concentration response curves for the effect of UCL1880 (■), UCL1495 (●) and glibenclamide (▲) on the response to 1 μM adenosine. Block was measured towards the end of an approximately 30 second application of adenosine to atrial cells held at -50mV. All inhibitors were applied for 4 minutes prior to the application of adenosine response. Values in brackets indicate the number of cells tested at each concentration of inhibitor.

COMPOUND	IC ₅₀ (μM)	n _H
UCL1880	0.18±0.02	0.95±0.07
UCL1495	0.46±0.04	1.68±0.35
Glibenclamide	23.5±5.50	0.78±0.18

Table 3.3.2: IC₅₀ and n_H values from the curves shown in Figure 3.3.2.

3.3.3: POSSIBLE USE-DEPENDENCE OF THE BLOCK BY UCL1880 AND UCL1495

Use-dependence has been described previously for UCL1495 (Jones 1995). As has been shown in Figure 3.3.1.1B the adenosine response shows a marked fade in the presence of 0.2μM UCL1880. This suggests that at low concentrations of blocker the current is activated before it is blocked i.e. the blocking action is use-dependent.

The effect of glibenclamide on the adenosine-induced current is quite different as already noted. In contrast to the blocking actions of UCL1495 and UCL1880 both the peak and the plateau response was reduced in the presence of glibenclamide and it would appear, therefore, that the blocking action of glibenclamide does not require the current to be activated. To quantify this, the effects of the drugs on the peak and steady state current were measured. Figure 3.3.3.1 illustrates the procedure.

The peak response and the steady state current level towards the end of the adenosine response, i.e. approximately 25 seconds after the initiation of the outward current, were measured. The ratio of steady state to peak was then calculated for both control adenosine responses and adenosine responses in the presence of the blocking drug. Figure 3.3.3.2. shows the results obtained. The change in the ratio with increasing concentrations of UCL1880 is illustrated in A. In control conditions where there is no UCL1880 present the ratio is 0.7± 0.1 (n= 21). In increasing concentrations of UCL1880 the ratio decreases indicating that UCL1880 causes a proportionately greater reduction in the steady state than

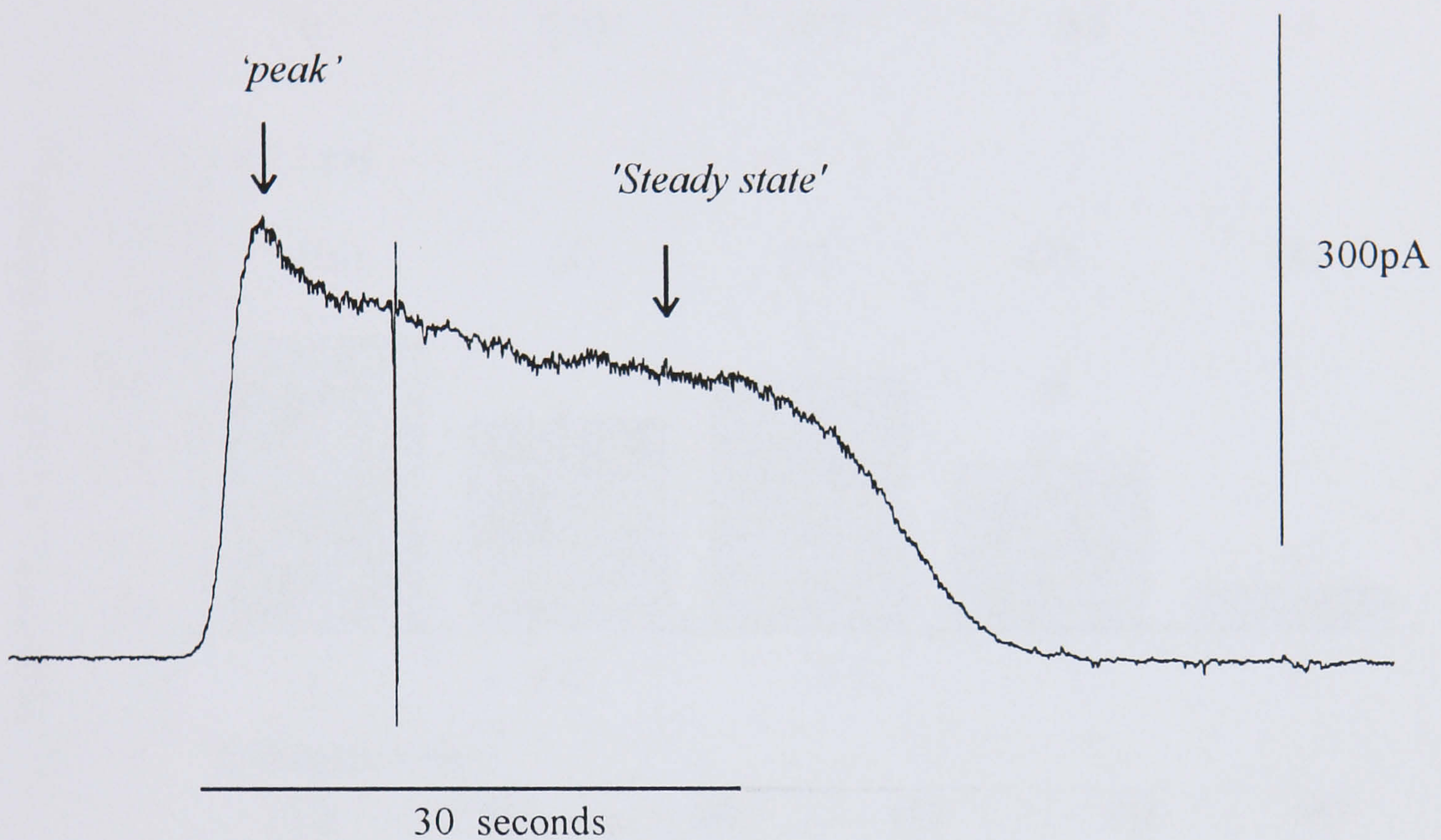


Figure 3.3.3.1: Measurement of peak and near steady state currents during an adenosine response. The steady state was divided by the peak current to obtain a ratio for the adenosine response in the absence and in increasing concentrations of blocking compound. If the steady state current is maintained the ratio will be close to 1. For a use dependent block, the ratio should get smaller compared with the control.

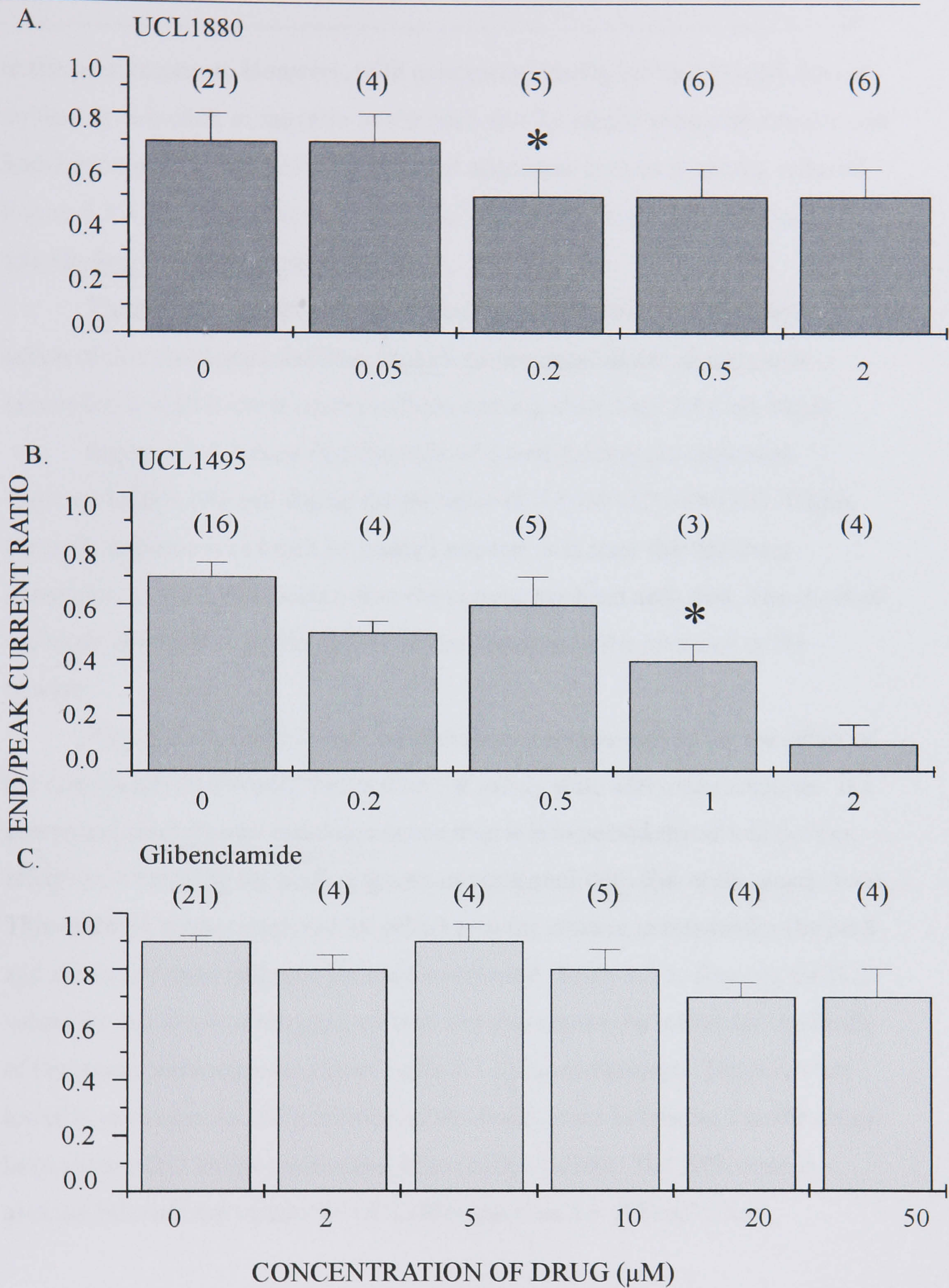


Figure 3.3.3.2: Effect of drugs on end/peak ratio of adenosine response. The steady state to peak current ratio was calculated in the absence and then in the presence of the indicated concentration of drug. Stars indicate a significant difference ($p < 0.05$) between control ratio and the ratio in the presence of the drug. The bars represent the standard error of the mean. The n number is shown above each bar in parenthesis.

in the peak response. However, with concentrations higher than $0.2\mu\text{M}$ there is no further reduction in the ratio as the peak and the steady state currents are now both becoming much smaller i.e. the total adenosine current is greatly reduced. Figure 3.3.3.2 B shows the effect of UCL1495, and at high concentrations of this blocker the ratio becomes small.

The effect of glibenclamide is illustrated in section C of the Figure. The action of this compound displays little or no use-dependence as the ratios remain fairly high even at concentrations causing more than 50% inhibition.

Figure 3.3.3.3 shows an example of a well maintained adenosine response before, (A) and during the presence of $0.5\mu\text{M}$ UCL1880 (B). Whilst the peak response is reduced by a small amount, it is clear that the main component of the block occurs after the current has been activated. The onset of the block can be seen in the decline of the response in the presence of the blocker.

Figure 3.3.3.4 shows log concentration-response curves for the effect of the compounds on the peak rather than the steady state adenosine response. If a compound displays any use-dependence then it is expected that it will be less effective in reducing the peak response as compared with that at the steady state. This could be further analyzed by calculating the relative potencies for the peak and the steady state response for each compound. Table 3.3.3. displays the IC_{50} values for the block of the peak current and also shows the values for the block of the steady state response (from Table 3.3.2) for comparison. The IC_{50} 's are lower in each case for the inhibition of the steady state indicating that the drugs have more effect on the maintained phase of the current. The difference is greatest between the values for UCL1880 and least for glibenclamide.

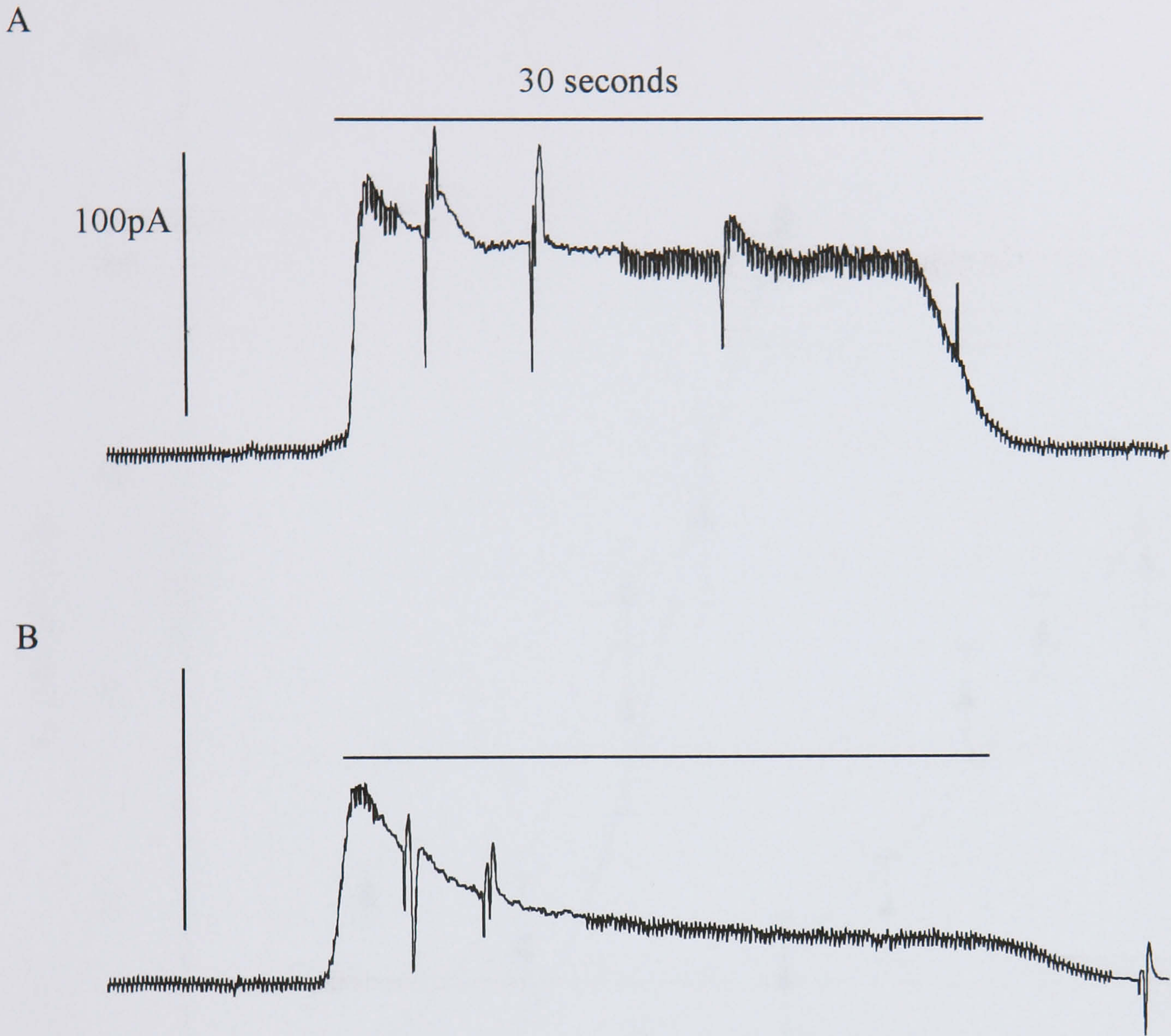


Figure 3.3.3.3: The use dependent effect of UCL1880 on the adenosine induced current. A; A control response to 1 μ M adenosine in a rat atrial myocyte. The peak declines to a maintained steady state level. After preincubation in 0.5 μ M UCL1880, B, the maintained current sags away to a much greater extent. The cell was held at -50mV. Adenosine was applied for 30 seconds. UCL1880 was preapplied for 4 minutes. Application bars are shown above each trace.

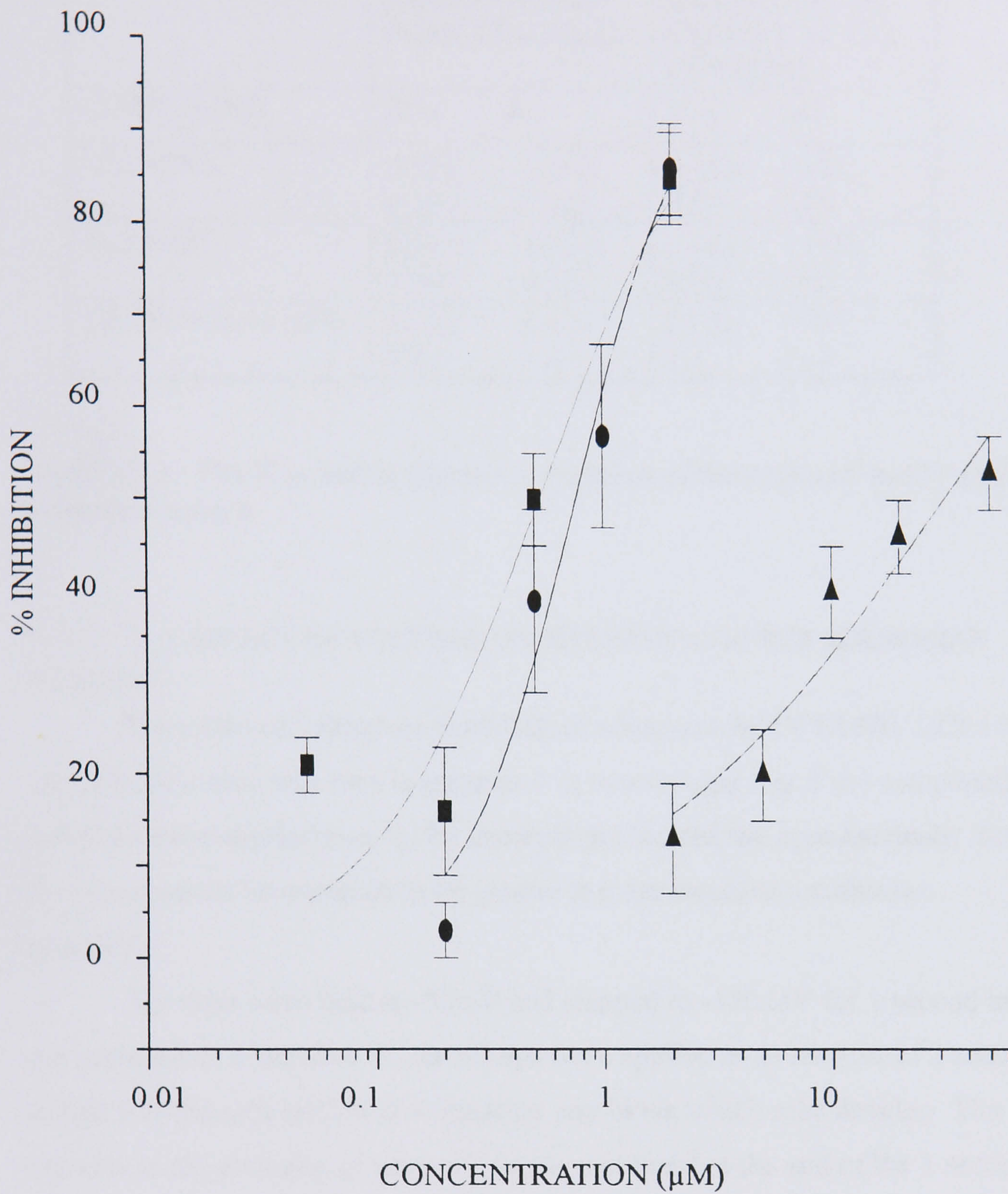


Figure 3.3.3.4: Dose response curves showing the effect of UCL1880 (■), UCL1495 (●) and glibenclamide (▲) on the peak adenosine response in atrial myocytes. The Hill equation was fitted to the data. The blockers were applied 4 minutes prior to adenosine application.

	INHIBITION OF PEAK CURRENT		INHIBITION OF STEADY STATE CURRENT	
COMPOUND	IC ₅₀	n _H	IC ₅₀	n _H
UCL1880	0.52± 0.16	1.16± 0.42	0.18± 0.02	0.95± 0.07
UCL1495	0.76 ± 0.09	1.73 ± 0.36	0.46± 0.04	1.68± 0.35
GLIBENCLAMIDE	31.3± 7.9	0.61± 0.6	23.5± 5.5	0.78± 0.18

Table 3.3.3 : The IC₅₀ and n_H values for the block of the peak and steady state adenosine current.

3.3.4: THE EFFECT OF VOLTAGE ON THE BLOCK OF THE ADENOSINE CURRENT.

The effect of voltage on the block of adenosine by UCL1880, UCL1495 and glibenclamide was next investigated. It was thought that if the compounds owed their use-dependence to, for example, a block of the open channels, then the block would be expected to be greater at more negative membrane potentials.

The cells were held at -50mV and stepped to -120 mV for 1 second in the presence of adenosine. 10 such steps were applied at an interval of 2 seconds as this was thought sufficient to measure any block which may develop. The currents in the presence of adenosine were measured at the end of the 1 second pulse. This was repeated after a 4 minute exposure to the drug. In all cases the background currents in the absence of adenosine were subtracted from the current with adenosine present.

Figure 3.3.4.1A shows the current produced by a step to -120mV in the presence of adenosine. Adenosine has induced an outward current at the holding potential of -50mV and an inward current in response to the step to -120mV. Following the very rapid development of the ohmic current due to the unblock of Mg²⁺ from the channels. there is a further increase in inward current which

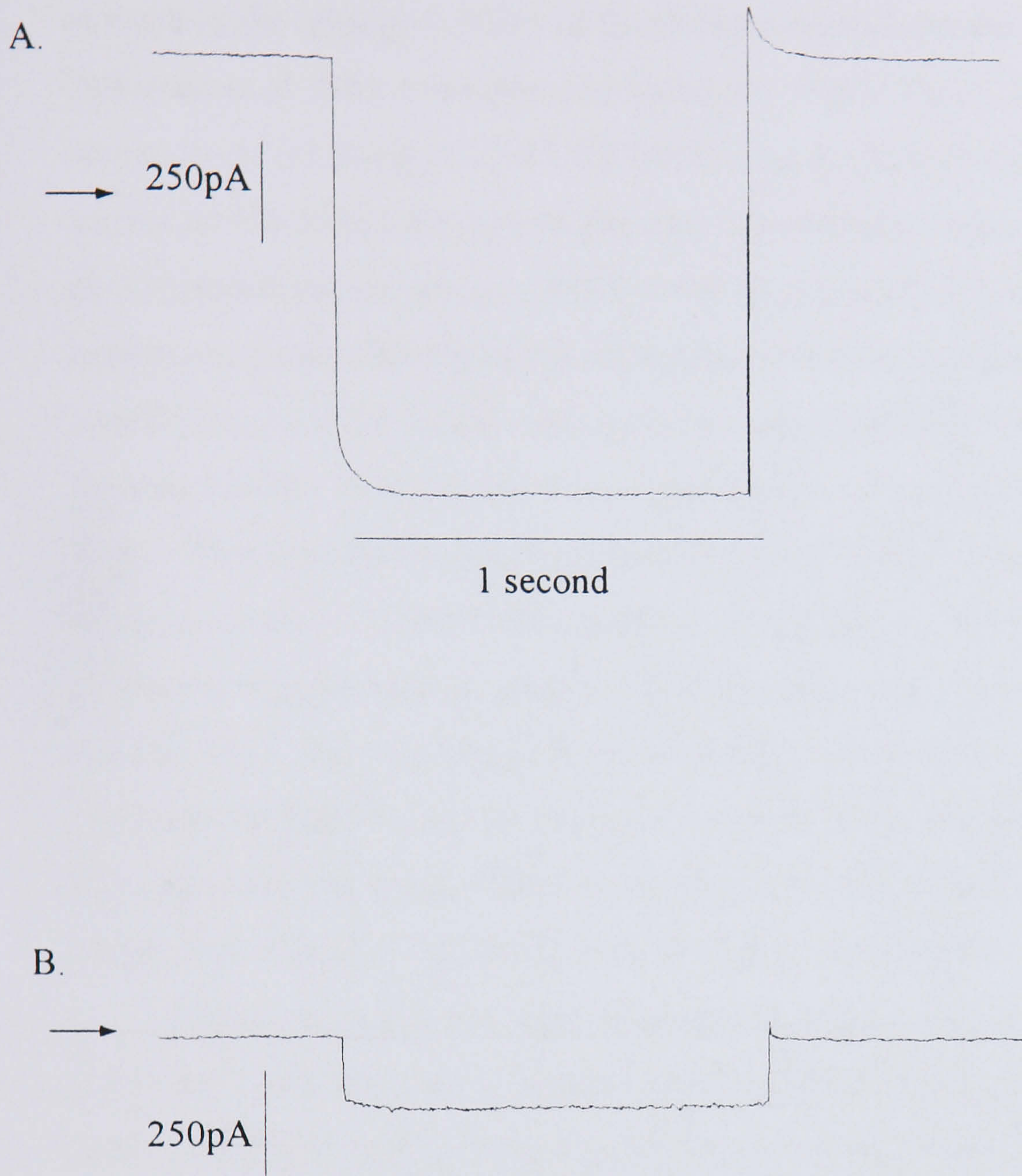


Figure 3.3.4.1. The effect of membrane potential on the block of the adenosine current. A. Current activated during a 1 second voltage step from -50 to -120 mV in the presence of 1 μM adenosine. B. The adenosine response in the presence of 2 μM UCL1880 applied 4 minutes beforehand. The arrows on the left indicate zero current level. Both the outward current at -50 mV and the additional current activated at -120 mV are blocked by the compound. The current traces shown are averages of 10 such jumps.

then remains at a steady state. This slow component is believed to be due to an increase in the open probability of the G-protein-gated channels.

(Sakmann et al 1983, Carmeliet and Mubagwa 1986). Figure 3.3.4.1B shows the current in the presence of 2 μ M UCL1880. It can be seen that the outward current at -50mV and the current pulse at -120mV have both been reduced. The time course of the current at -120mV in the presence of UCL1880 does not display any decay after the initial instantaneous current and both the instantaneous and the steady state level have been reduced. Note that on stepping back to -50mV in the control step there is an outward current which decays. This is probably a consequence of a relief of Mg²⁺ and polyamine block which occurred at -120mV and resulted in an increase in the outward holding current on stepping back to -50mV. The transient outward current decays as block by Mg²⁺ and polyamines is re-established. The transient outward current could also be partly due to the deactivation of the G-protein-gated channels (Carmeliet and Mubagwa 1986). In the presence of UCL1880 this transient current was reduced. This tail current was not studied further.

Figure 3.3.4.2 A shows the response of another cell in the presence of adenosine. After the initial inward current in response to stepping to -120mV there is a further increase in the inward current which, in this instance, then declines a little. This subsequent decline may be due to extracellular block by cations such as Na⁺ as is the case for background inward rectifiers (Kelly *et al.*, 1992, Voets *et al.*, 1996) or to desensitization of the G-protein coupled receptors. Figure 3.3.4.2B shows the adenosine response at -120mV in the presence of 20 μ M glibenclamide. Both the outward and inward currents have been reduced.

The results from all three compounds at -120mV are summarized in Table 3.3.4 which also shows the percentage inhibitions at -50mV. These results indicate that there is little voltage-dependence of the inhibition of the adenosine-induced current by any of the compounds. It is also noteworthy that, if there was an increase in block at -120mV, then the current would have possibly relaxed at this potential.

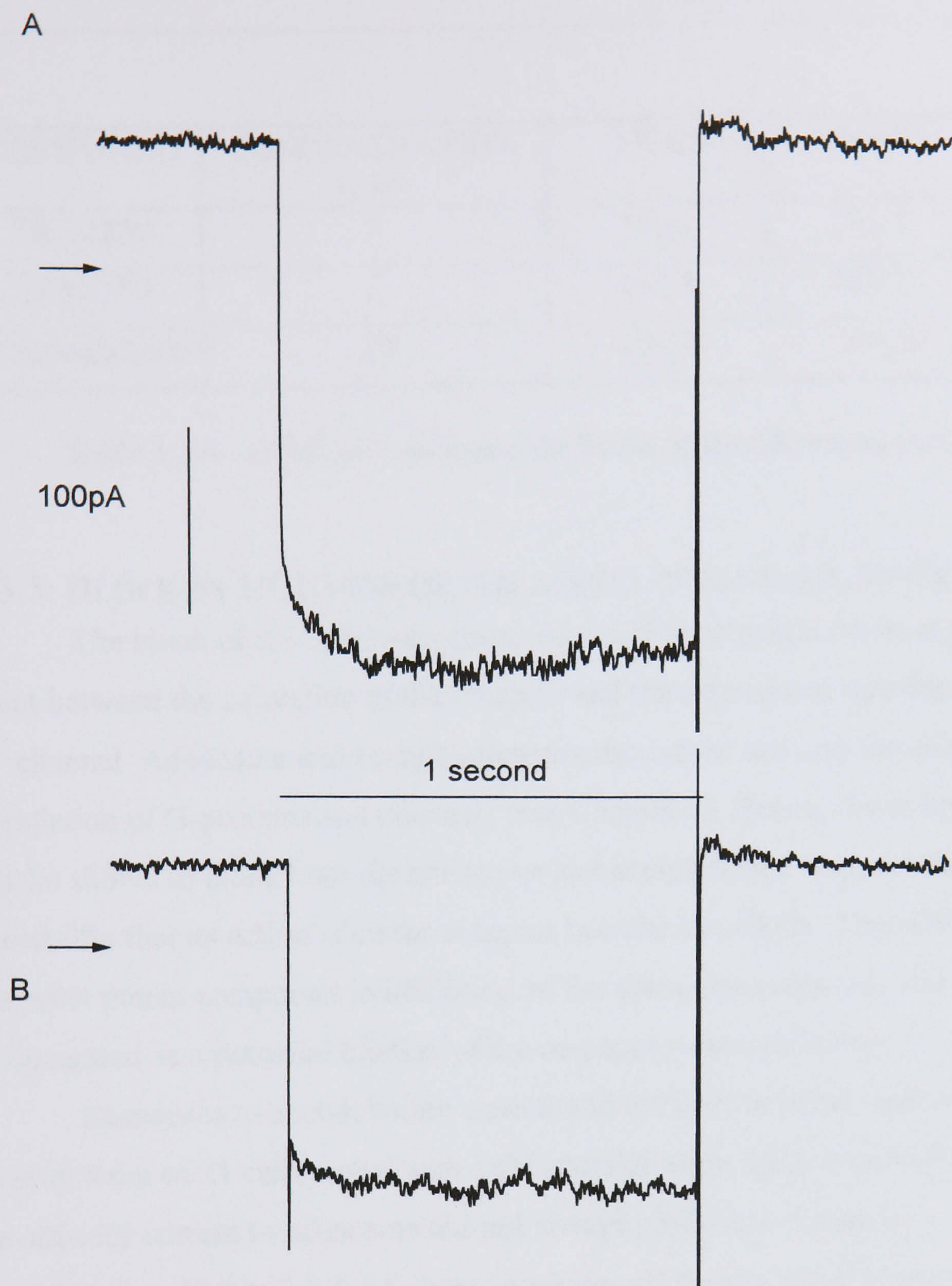


Figure 3.3.4.2: A shows the control current in response to a step from -50mV to -120mV in the presence of 1 μ M adenosine. B shows the current at -120mV in the presence of adenosine applied after a 4 minute incubation in 20 μ M glibenclamide. Glibenclamide was bath applied. In both traces there is a small inward current activated on returning to the holding potential of -50mV.

COMPOUND	CONCENTRATION (μ M)	-120mV	-50mV
UCL1880	2	86 \pm 4	78 \pm 5
UCL1495	2	69 \pm 5	78 \pm 8
Glibenclamide	20	35 \pm 7	34 \pm 9

Table 3.3.4 : Effect of voltage on the block of the adenosine current.

3.3.5: BLOCK BY UCL1880 OF THE ACETYLCHOLINE RESPONSE.

The block of the adenosine response could in principle occur at any point between the activation of the receptor and the subsequent opening of the K⁺ channel. Adenosine and acetylcholine are thought to activate the same population of G-proteins and channels (see Chapter 1). Hence, if a compound can be shown to block both the adenosine and acetylcholine response then the possibility that its action is on the receptor become less likely. Thus UCL1880, the most potent compound in inhibition of the adenosine response, was investigated as a potential blocker of the response to acetylcholine.

Responses to acetylcholine were found difficult to obtain and were seen in only 8 out of 21 cells tested with 1 μ M acetylcholine. Cells which developed an outward current to adenosine did not always produce a current to acetylcholine. Figure 3.3.5.1A shows a whole cell current trace from a cell bathed in 1 μ M acetylcholine which did result in an outward current. It usually differed from that to adenosine in that the peak and steady state phases were less clearly distinguished.

Figure 3.3.5.1B shows the effect of prior incubation with 0.2 μ M UCL1880. The extent of inhibition was calculated in the same way as in the experiments with adenosine. In 4 cells the average steady state inhibition caused by 0.2 μ M UCL1880 was 48 \pm 7%. This is consistent with the figure of 53 \pm 5% for inhibition of the adenosine response by the same concentration of UCL1880.

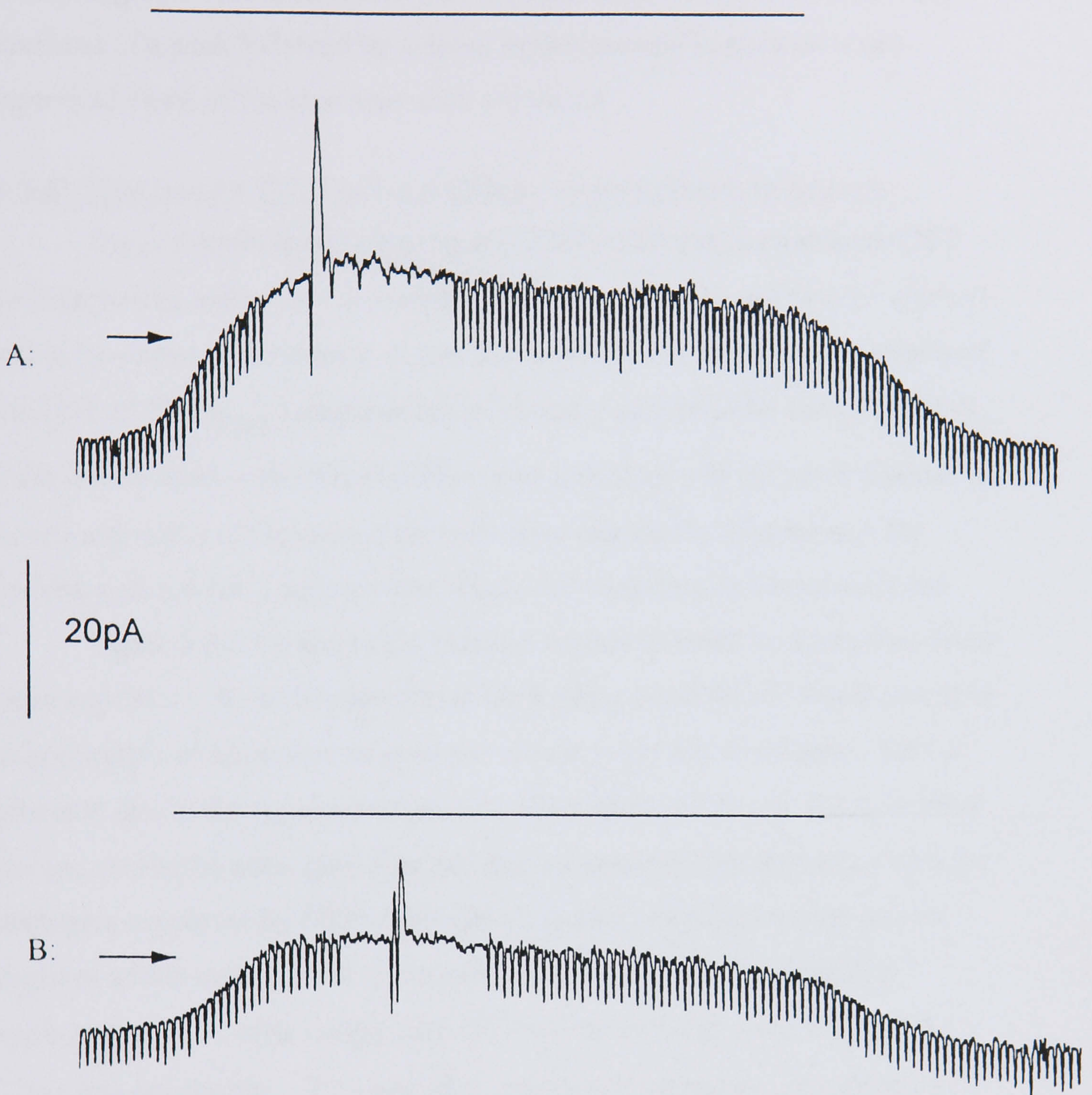


Figure 3.3.5.1: A shows a control whole cell current response to 1 μ M acetylcholine. B shows the acetylcholine response after a four-minute incubation in 0.2 μ M UCL1880. The cell was held at -50 mV and the drugs were bath applied. The downward deflections are due to 10 mV voltage steps. The larger deflections are due to the application of voltage ramps. The horizontal bars above the response indicate the 30-second application of acetylcholine.

Figure 3.3.5.2 shows an example of an ACh current which displayed a greater degree of desensitization. The response in the presence of UCL1880 consisted of a peak followed by a decay in the outward current i.e. a use-dependent block of the kind seen with adenosine

3.3.6: THE USE OF GTP- γ -S TO VERIFY NON-RECEPTOR BLOCK.

The non-hydrolyzable analogue of GTP, GTP- γ -S, activates the GTP binding protein and causes irreversible activation of the muscarinic K⁺ channel whilst by-passing the receptor. It was thus possible to investigate the effects of the inhibitors on I_{K_{ACh}} independently of receptor activation by using GTP- γ -S. Cells were loaded with 100 μ M GTP- γ -S by including it in the patch pipette. To ensure activation of the current the cells were exposed to adenosine in the bathing solution for 1 minute when whole cell recording had been achieved.

Figure 3.3.6.1A shows the outward current initiated by adenosine under these conditions. It can be seen that at the holding potential of -50mV and prior to application of adenosine an outward current is already developing. This is probably due to the agonist-independent effect of the GTP- γ -S. The additional current evoked by adenosine does not display as much fade compared with the responses supported by GTP. After the adenosine is washed out the current declines a little but not back to the control level (i.e. prior to adenosine application). In 37 cells loaded with GTP- γ -S an outward current of 106.4 ± 1 pA was maintained ~ 1 minute after washout of adenosine. The effects of 2 and 10 μ M UCL1880 are shown. 2 μ M UCL1880 produces a rapid and substantial decline in the outward current and increasing the concentration of UCL1880 from 2 to 10 μ M does not produce a significantly greater block.

The upper trace in Figure 3.3.6.1B shows the adenosine response from a different cell and again a slight outward current develops prior to application of adenosine. After adenosine washout the current declines to a lower maintained level. The effect of 1 μ M glibenclamide was then tested. At this concentration there was little block.

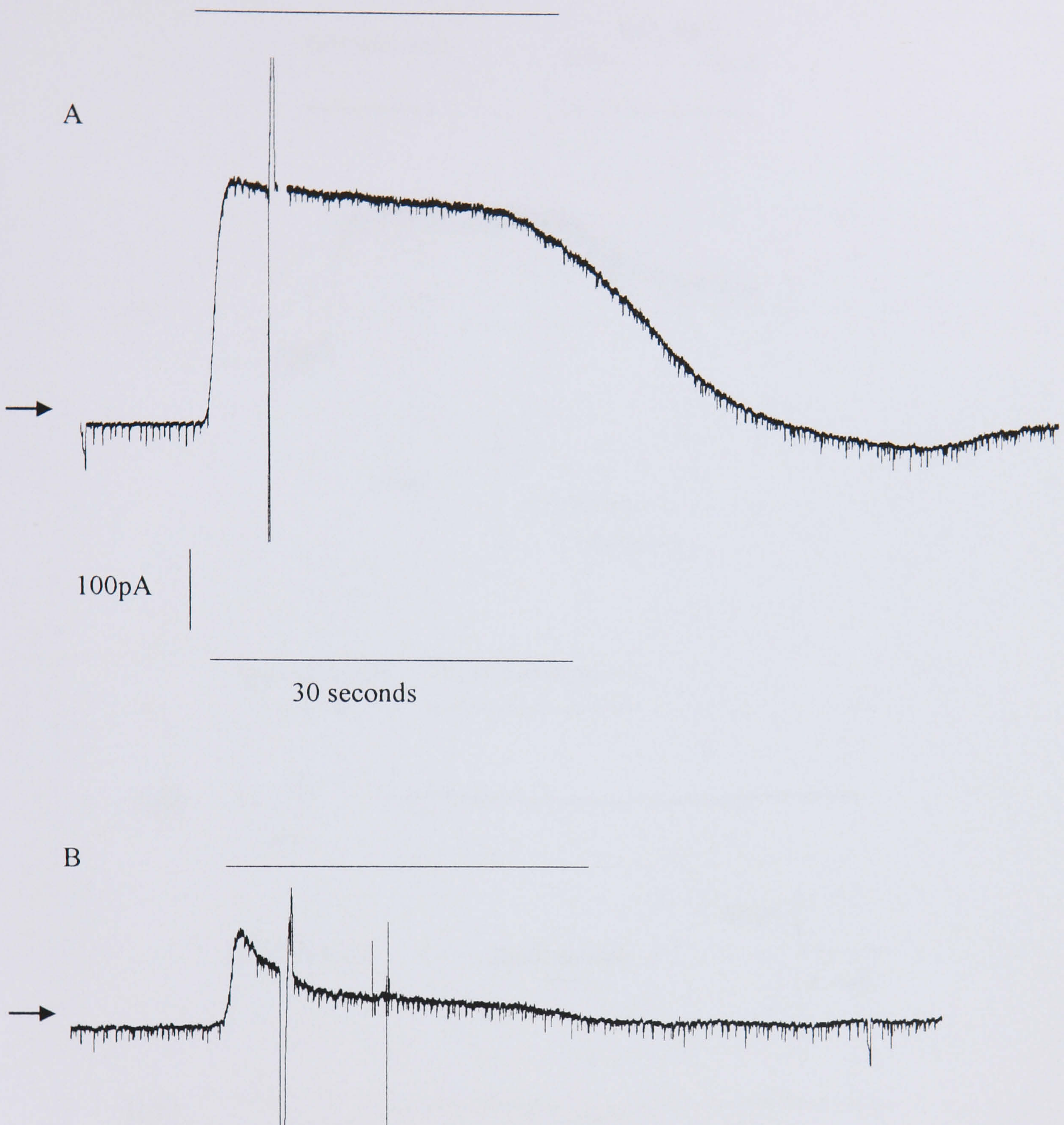


Figure 3.3.5.2. The effect of UCL1880 on the response to 1 μ M acetylcholine in a neonatal rat atrial myocyte. The cell was held at -50 mV. Acetylcholine, 1 μ M, was bath applied for 30 seconds, as indicated by the horizontal bars above each trace. A; A control acetylcholine response. B; the response in the presence of 2 μ M UCL1880 after a 4 minute preincubation period. Note the sag of the response in the presence of UCL1880. This is as for adenosine. Downward deflections are due to -10 mV voltage steps and bigger deflections across the trace are due to voltage ramps. Note that the 'spikes' are not very well reproduced from the chart recorder tracing.

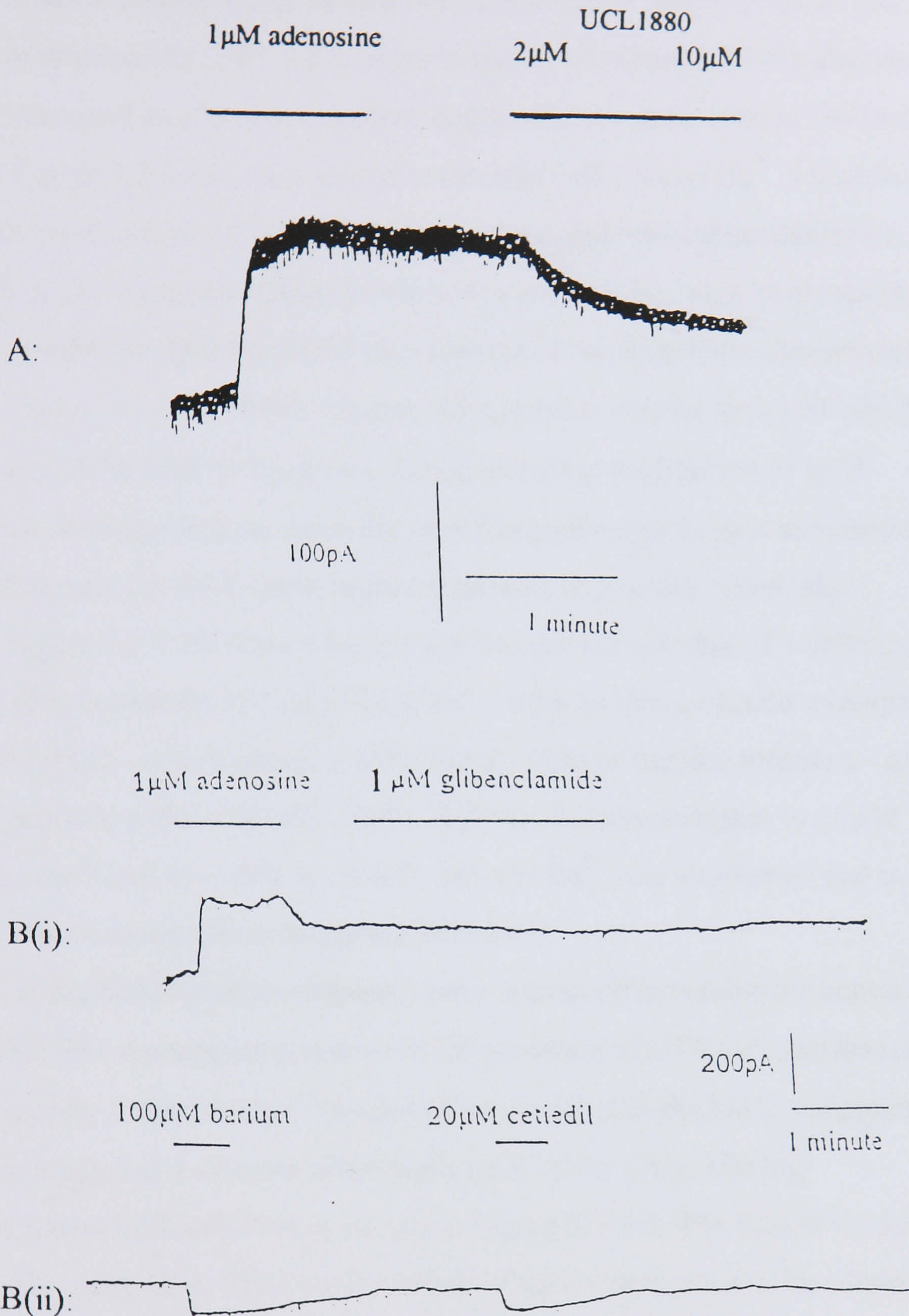


Figure 3.3.6.1: A shows the outward current due to 1 μ M adenosine and the maintenance of this current by 100 μ M GTP- γ -S in the patch pipette. 2 and 10 μ M UCL1880 were applied cumulatively as indicated and reduced the current. B (i): The maintenance of an outward current initiated by application of adenosine. 1 μ M glibenclamide has little effect; (ii) shows the effect of 100 μ M barium and 20 μ M cetiedil. The time and current scales are different for A and B. Some outward current develops prior to adenosine application. Cells were held at -50 mV, and all drugs were bath applied.

It has been previously shown that cetiedil and UCL1495 block the current maintained by GTP- γ -S current in these cells (Jones, 1995). Therefore cetiedil was used as a 'positive control' and its effect can be seen in the lower trace in Figure 3.3.6.1B. Also shown is the effect of 100 μ M Ba²⁺ which has been also used as a positive control. Both compounds caused an inhibition which is quick in onset but appears slower in offset. This most likely reflects a delay in washout rather than slow dissociation of the drug from the channels.

Figure 3.3.6.2A shows current-voltage relationships for a cell which has been loaded with GTP- γ -S prior to, during and after application of 1 μ M adenosine. The currents are inwardly rectifying although there is substantial outward current for the G-protein-gated currents at positive potentials.

Figure 3.3.6.2B shows the current-voltage relationship of a different cell and the effects thereon of 2 and 10 μ M UCL1880. In this particular example 2 μ M UCL1880 - which caused \sim 90% block of the adenosine response - can be seen to produce a block of only \sim 20%. Raising the concentration to 10 μ M increases the block to \sim 50% at -50mV. 100 μ M Ba²⁺ was also tested and is observed to produce almost complete inhibition.

A log concentration-response curve was constructed for the action of UCL1880. The maintained response in the presence of GTP- γ -S was measured approximately 1 minute after washout of adenosine and the blocked response was then measured 4 minutes after application of the drug. The log concentration-response curve is shown in Figure 3.3.6.3. The IC₅₀ of 0.25 μ M is close to the value of 0.18 μ M for the block of the current induced by adenosine. However the maximum and the slope are different. On the adenosine-induced current 2 μ M UCL1880 causes an inhibition of 90% (Figure 3.3.2) as compared with 55% in GTP- γ -S loaded cells. Raising the concentration to 10 μ M does not further increase the block observed. The reduction in the maximum suggests that only a component of the GTP- γ -S current is inhibited by UCL1880. It may well be that the inclusion of current carried by another ion which is insensitive to UCL1880. One possibility is that the additional activated current is through the K_{ATP} channels. However, the small effect of glibenclamide on this current does not support this as 1 μ M glibenclamide is very potent on the K_{ATP} channels

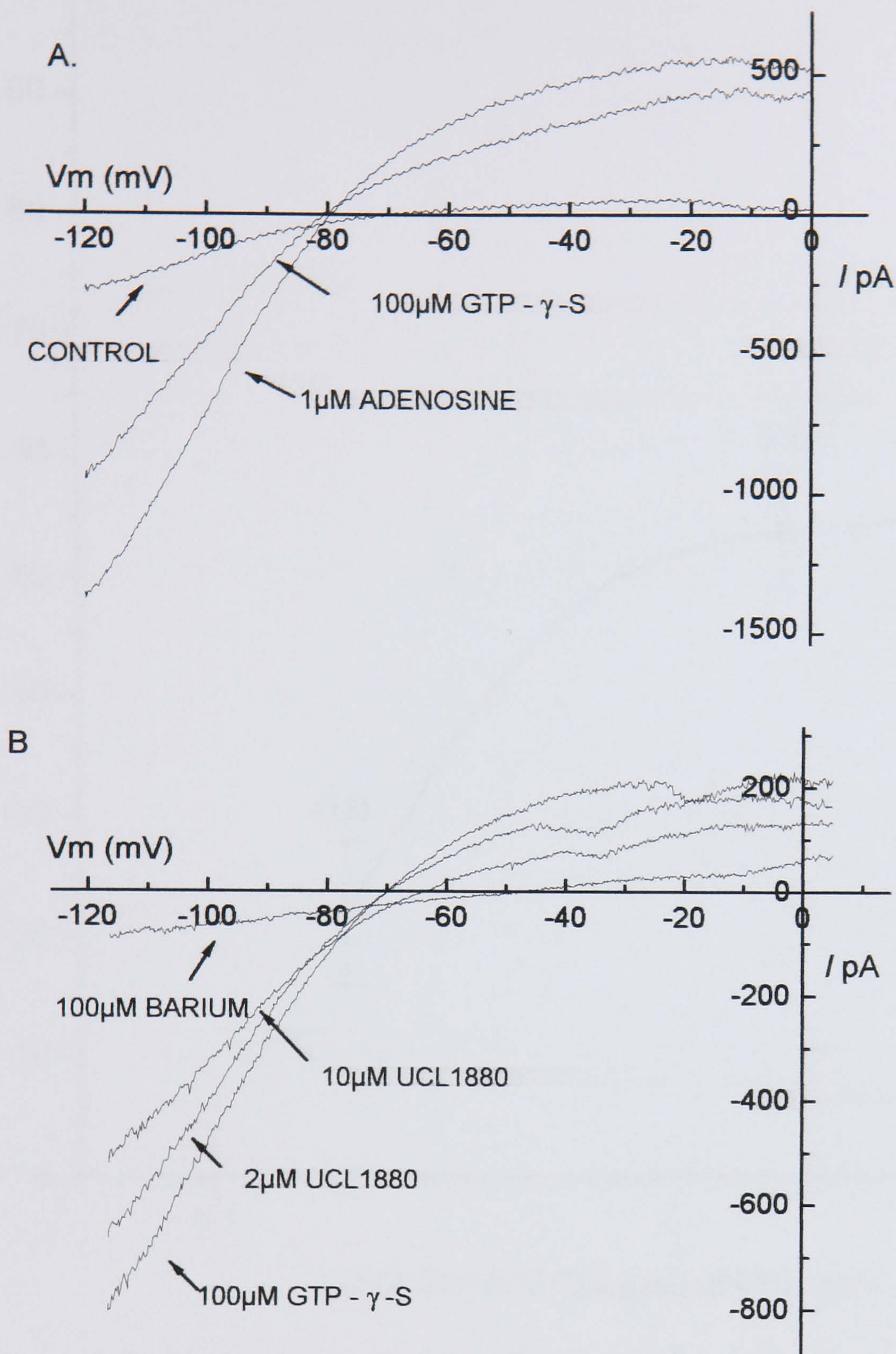


Figure 3.3.6.2: Current- voltage relationship of the GTP- γ -S current in atrial myocytes. A displays the control curve, the current activated by 1 μ M adenosine, and the current maintained by GTP- γ -S. B displays the effect of 2 and 10 μ M UCL1880 and 100 μ M barium. Cells were held at -50mV and ramped from between -120 and 0mV. 100 μ M GTP γ - S was included in the patch pipette.

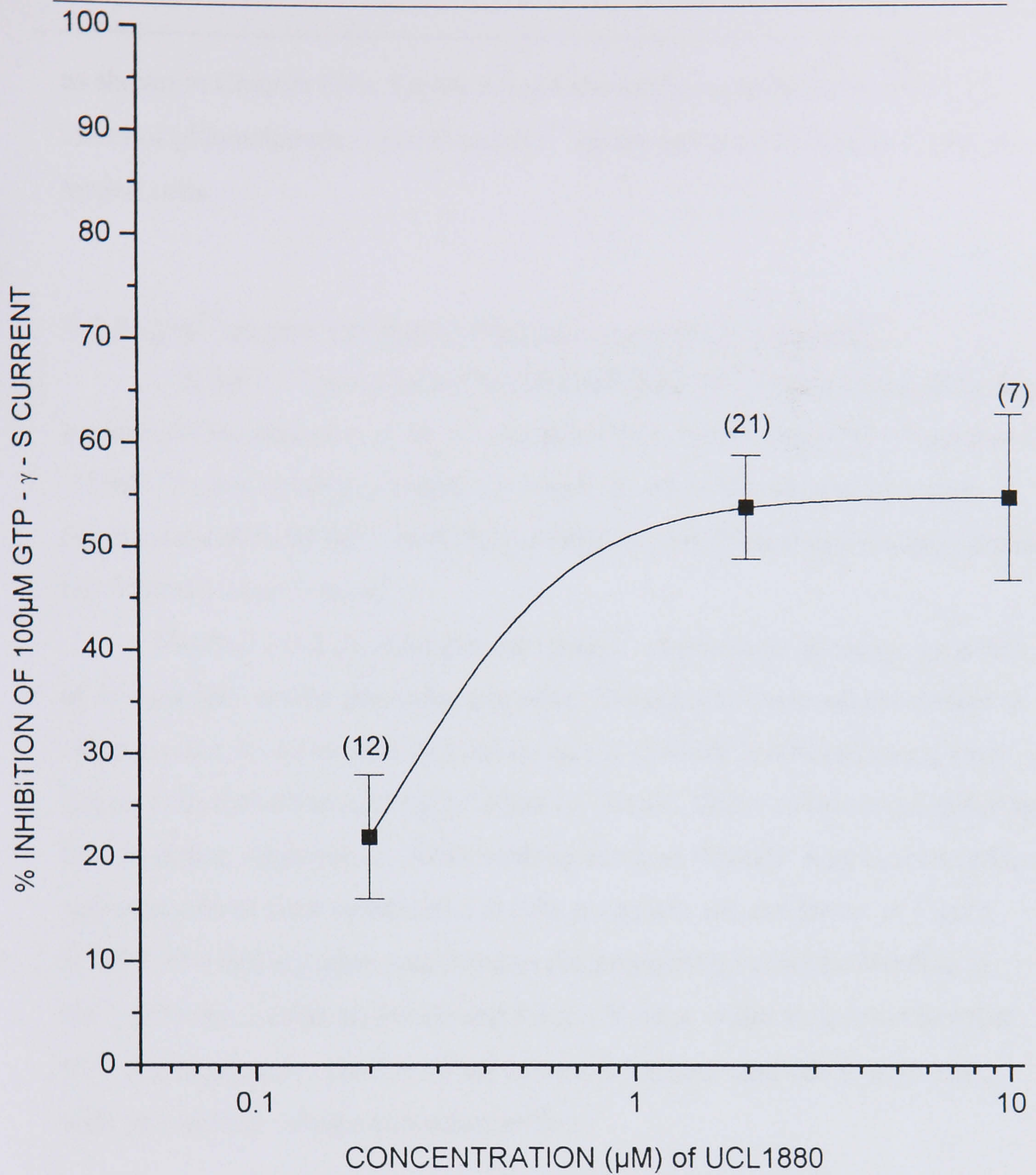


Figure 3.3.6.3: UCL1880 block of the GTP- γ -S current in rat atrial myocytes. 1 μ M adenosine was applied to activate the current. Data were fitted with the Hill equation giving an IC_{50} value of 0.25 μ M and a Hill slope of 1.89. The maximum was reduced to 55%. Values above each data point indicate the number of cells tested with that concentration of inhibitor.

as shown in Chapter Five. Figure 3.3.6.4 shows the combined results for the effect of glibenclamide, cetiedil and Ba^{2+} on the current activated in GTP- γ -S loaded cells.

3.3.7: Ba^{2+} BLOCK OF THE G-PROTEIN-GATED K^+ CHANNEL

Figure 3.3.7.1 illustrates the effect of $3\mu\text{M}$ Ba^{2+} on the current flowing in response to adenosine. A shows the adenosine current during a voltage step to -120mV from a holding potential of -50mV . B shows the adenosine current in the presence of $3\mu\text{M}$ Ba^{2+} ; both the outward (at -50mV) and the inward currents (at -120mV) were reduced.

Figure 3.3.7.2 presents current-voltage relationships showing the effect of $100\mu\text{M}$ Ba^{2+} on the adenosine response. Though Ba^{2+} reduced the current at all potentials, it can be seen that the current at -120mV is blocked to a greater degree than that at the holding potential of -50mV . This was investigated further by calculating the block at -50mV and the block at -120mV . Log concentration-response curves were constructed at both potentials and are shown in Figure 3.3.7.3. The Hill equation was fitted to the data giving values for the IC_{50} of Ba^{2+} of $34.9 \pm 2.2\mu\text{M}$ at -50mV and $3.8 \pm 1.8\mu\text{M}$ at -120mV . It is evident that Ba^{2+} is a more active blocker of the current at -120mV and this is in keeping with its reported voltage-dependent action.

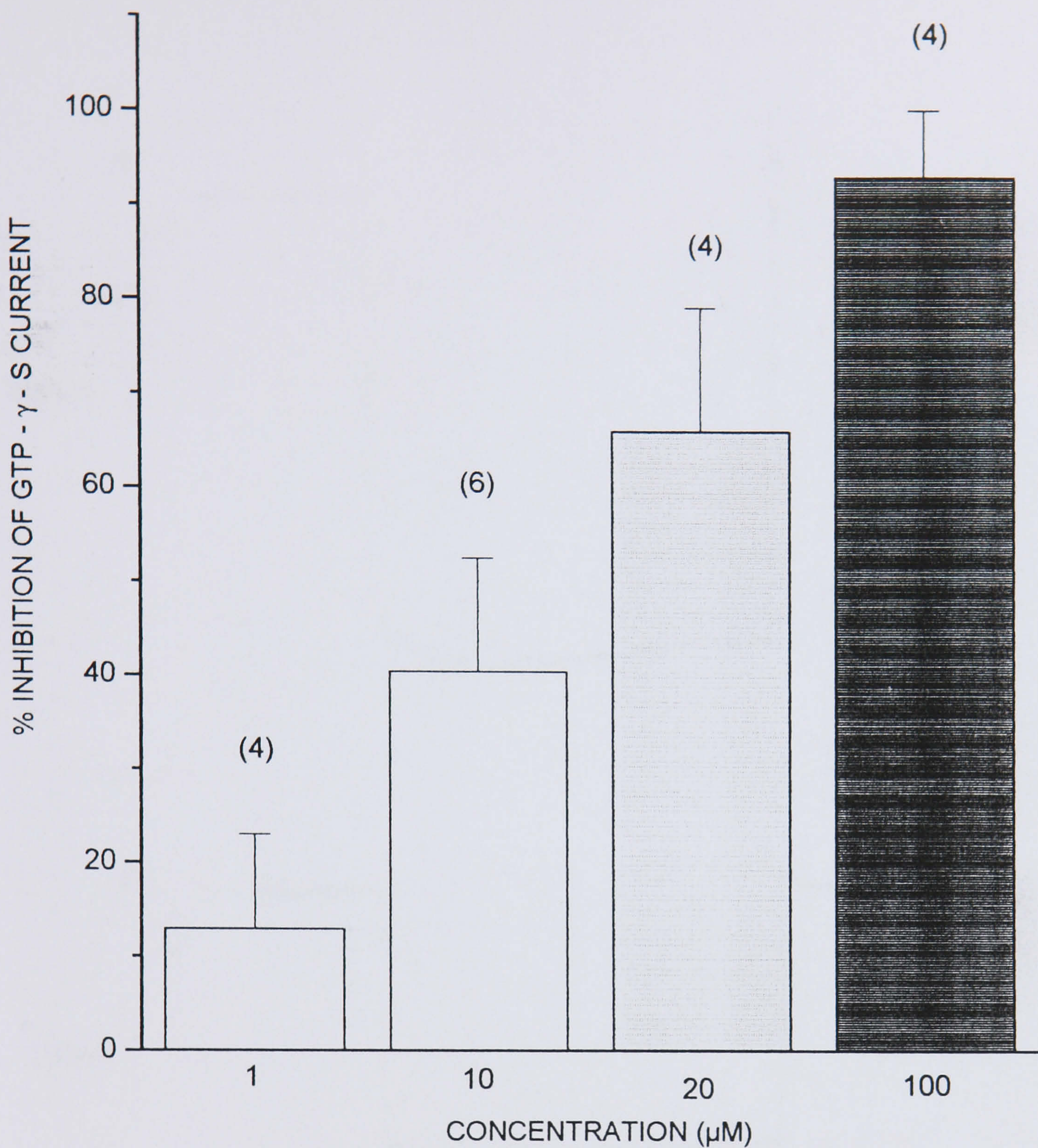
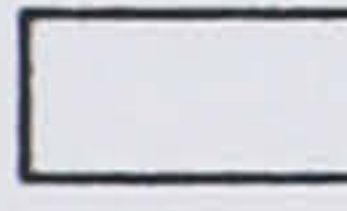
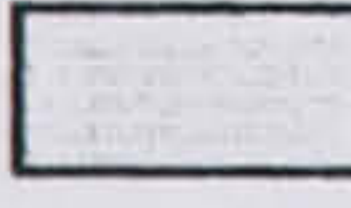



Figure 3.3.6.4: A summary of the results from experiments carried out using GTP-γ-S. The results for the effects of glibenclamide () at 1 and 10 μM, cetiedil () and barium () are shown. Values in brackets above each column indicate the number of cells used in the experiments.

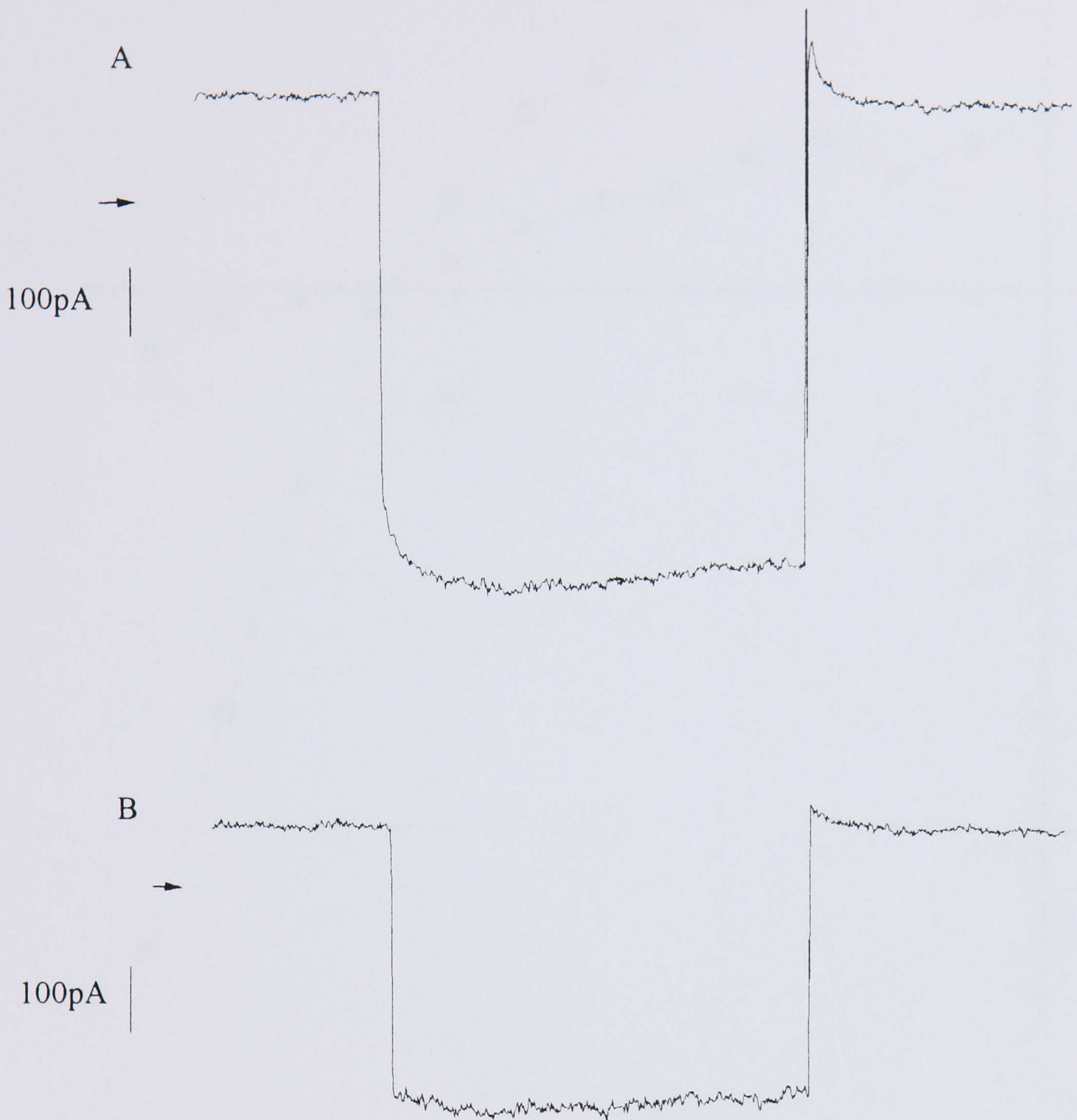


Figure 3.3.7.1: The effect of 3 μ M barium on the current in the presence of 1 μ M adenosine at -120 mV. A shows the control current at -120 mV. B shows the current in the presence of adenosine after the cell was incubated in barium for 4 minutes. Both the inward and outward currents have been reduced. The current at -120 mV has declined by approximately 50%. Cells were held at -50 mV and stepped down to -120 mV for 1 second.

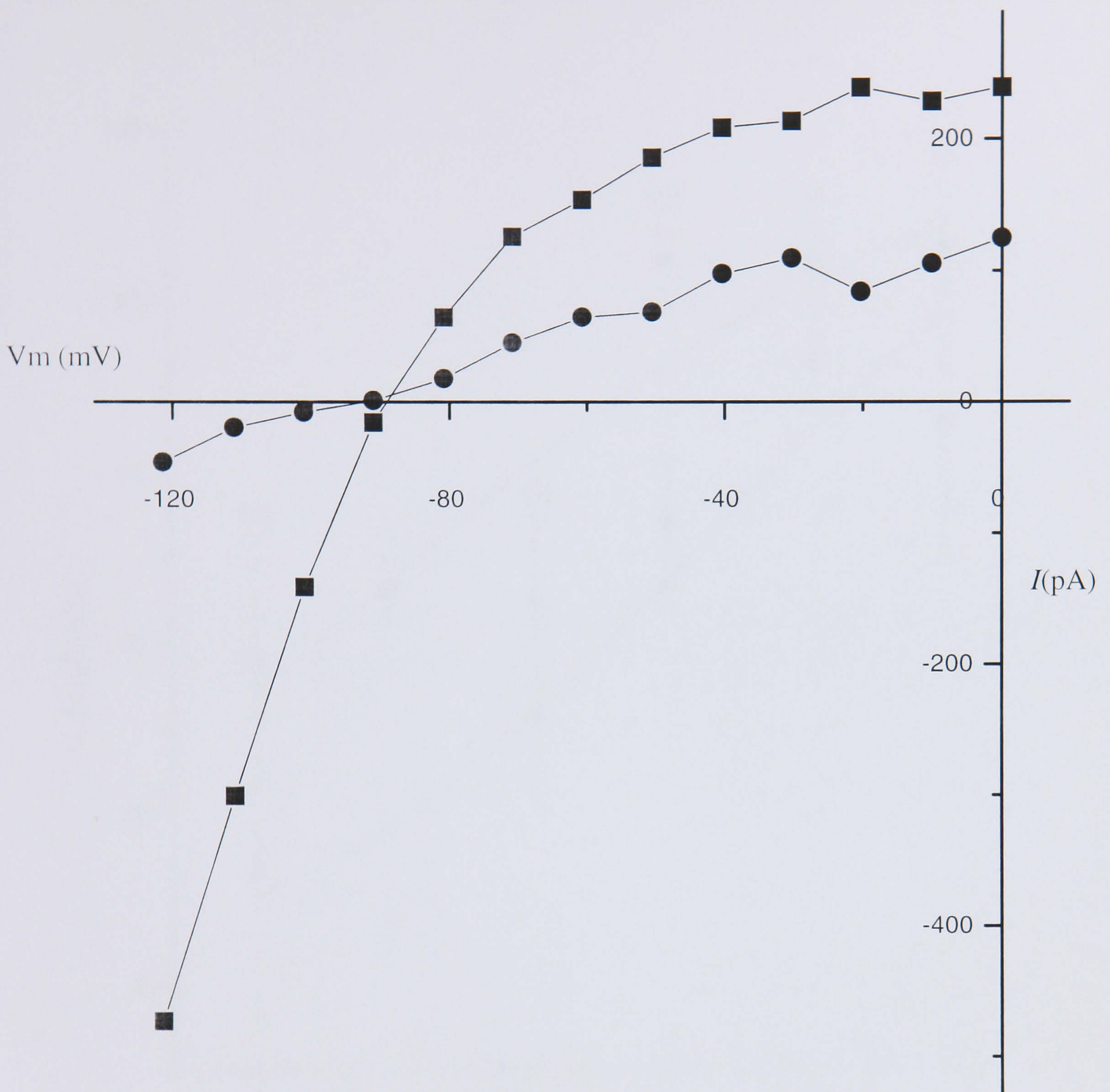


Figure 3.3.7.2: The current in the presence of 1 μ M adenosine (—■—), and the effect of 100 μ M barium (—●—) thereupon are shown. The cell was held at -50 mV and stepped from between approximately -120 and 0 mV in +10 mV increments. Currents were measured at the steady state in each pulse.

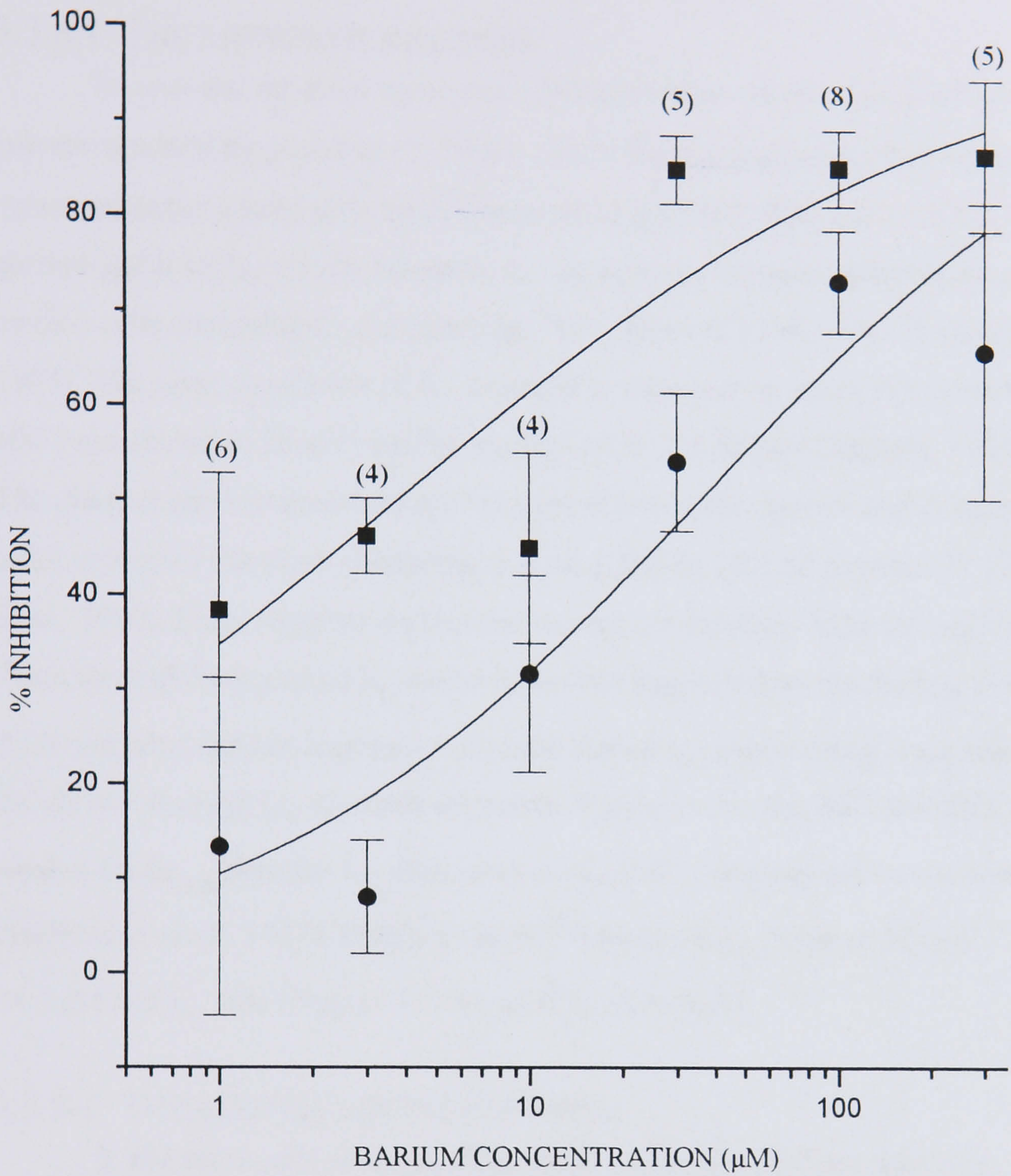


Figure 3.3.7.3: Log-concentration response curve for barium block of $I_{K ACh}$. Block of the outward current have been measured at -50mV (\bullet) and of the inward current at -120mV (\blacksquare). Data points were fitted with the Hill equation. The numbers of cells tested for each concentration are given in brackets above each point.

3.3.8: DISCUSSION:

3.3.8.1: THE ADENOSINE RESPONSE:

In neonatal rat atrial myocytes 1 μ M adenosine activated an outward current at a holding potential of -50mV. Its amplitude was 162 ± 23 pA when measured at the steady state level. The reversal potential shows it to be a K^+ current and it is likely to be the same as that activated by acetylcholine in cardiac cells (Belardinelli and Isenberg, 1983, Kurachi *et al.*, 1983, Ito *et al.*, 1995). The same population of K^+ channels in neonatal rat atrial myocytes has also been shown to be activated by somatostatin (Lewis and Clapham, 1989). The channel conductance does not vary greatly between species and in human atrial myocytes the slope conductance is 46 ± 2 pS in 145mM external K^+ (Sato *et al.*, 1990). Early suggestions that the current activated by ACh and adenosine flows through background I_{K1} channels are not supported by the finding that the ACh-activated current increases with time during hyperpolarising steps whereas the current through I_{K1} channels decreases. Furthermore, the Ba^{2+} sensitivity is weaker for $I_{K_{ACh}}$ than for I_{K1} (Sakmann *et al.*, 1983, Soejima and Noma 1984, Heidbüchel *et al.*, 1987). In this study Ba^{2+} blocked $I_{K_{ACh}}$ with an IC_{50} of 34.9 μ M and I_{K1} (see Chapter 4) with an IC_{50} of 16.8 μ M.

3.3.8.2: THE ACETYLCHOLINE RESPONSE.

In the same cells acetylcholine, unlike adenosine, did not routinely activate an outward current, which was seen in only 8 out of 21 cells tested. In guinea-pig atrial cells the EC_{50} for the ACh response is 0.15 μ M (Kurachi *et al.*, 1986) so that the concentration of 1 μ M ACh used in this study should have been sufficient. The presence of muscarinic receptors in atrial tissue of the rat has been demonstrated (Wei and Sulakhe, 1978, Dashwood and Spyer, 1986) and it has also been shown that there is a muscarinic current present in fetal and neonatal rat hearts (Takano and Noma, 1997). There is no obvious explanation for the lack of response observed in the present work. It is possible that not enough ACh receptors are activatable under the conditions of my experiments.

3.3.8.3: DESENSITIZATION:

The response to adenosine showed 'fade' in that the outward current reached a peak and then declined to a lower maintained level. The process is often described as desensitization and has been observed in many studies in cardiac tissue (Carmeliet and Mubagwa, 1986, Heidbuchel *et al.*, 1987, Kurachi *et al.*, 1987, Kim 1991, 1993). Desensitization has been reported to be biphasic in that there is an initial phase lasting ~20 seconds followed by a slower phase lasting several minutes (Zang *et al.*, 1993, Kim, 1993), this was not analyzed in the present work. Desensitization is seen both with acetylcholine and adenosine. Also an acetylcholine response cannot be elicited after desensitization induced by adenosine suggesting a common mechanism underlying the desensitization seen with both agonists (Kurachi *et al.*, 1987). This heterologous desensitization suggests an effect at the G protein and/or channel level. The desensitization is seen on activating the current either with agonists or with GTP- γ -S and thus by-passing the receptor (Zang *et al.*, 1993). With agonist activation both fast and slow phases of desensitization are seen whereas with the GTP- γ -S the slow phase of desensitization is less obvious. Thus the fast phase of desensitization is thought to be due to a decline in the activity of the G protein and/or the K⁺ channel whereas the slow phase may represent a reduction in the receptor activity. The early phase of desensitization is associated with a shortening of channel open time and increase of mean closed time. This is possibly due to the action of a Ca²⁺-calmodulin dependent phosphatase, since the addition of [ATP]_i increases the mean open time and this effect of ATP is reversed by increasing intracellular Ca²⁺ (Kim 1991). The ACh-activated channel can, therefore, be modulated by phosphorylation and dephosphorylation.

3.3.8.4: BLOCK OF THE ADENOSINE RESPONSE:

The results presented in this chapter show that UCL1880, UCL1495, glibenclamide and Ba²⁺ are effective in blocking the G protein-gated K⁺ current activated by adenosine in neonatal rat atrial myocytes. UCL1880 was the most potent inhibitor with an IC₅₀ of 0.18 μ M. The structural analogue of cetiedil,

UCL1495 was also quite active blocking the current in the low μM range. Glibenclamide, which is a very potent inhibitor of $I_{K_{ATP}}$, also blocked the current though only at higher concentrations than for UCL1880 and UCL1495.

3.3.8.4.1: UCL1880 AND UCL1495 BLOCK OF THE ADENOSINE CURRENT.

It has been reported that the block by clotrimazole of the G-protein-gated K^+ current activated by acetylcholine and adenosine in atrial myocytes is use-dependent (Jones, 1995) in the sense that the adenosine response faded more rapidly in the presence of clotrimazole. In the present study the block caused by the structural analogue of clotrimazole, UCL1880, showed the same feature (see Figure 3.3.1.1 and 3.3.3.3). This has been quantified for UCL1880 in Figure 3.3.3.2.

UCL1880 was effective in blocking the response to acetylcholine as well as to adenosine reducing the likelihood that the block involved an interaction with the receptor.

If, as these results suggest, the block occurs at the G-protein and/or K^+ channel stage and not at the receptor then activation of the current independently of receptor activation by GTP- γ -S should also be inhibited by UCL1880. In such experiments UCL1880 did indeed cause a block, though not as much as observed for the action of UCL1880 on the agonist-induced current. It might be argued that this was because UCL1880 has caused some receptor block which contributes to the overall inhibition of the adenosine-induced current, this component being absent in the GTP- γ -S current. An important observation, however, was that UCL1880 could not produce complete inhibition of the GTP- γ -S-activated current. As shown in Figure 3.3.6.1 and Figure 3.3.3.6, increasing the concentration of UCL1880 from 2 to $10\mu\text{M}$ did not substantially further increase the amount of block observed. The IC_{50} value for the block by UCL1880 of the GTP- γ -S current was estimated at $0.25\mu\text{M}$ which is close to the value of $0.18\mu\text{M}$ for the block of the adenosine-induced current. The maximum block of $\sim 55\%$ by UCL1880 of the GTP- γ -S current is

achieved at between 2 and 10 μM and there remains a UCL1880 insensitive component. This is blocked by 100 μM Ba^{2+} and thus may be a K^+ current. In the light of these observations it can be speculated that the inclusion of GTP- γ -S in the patch pipette has resulted in the activation of not only $\text{I}_{\text{K}_{\text{ACh}}}$ but also an additional current. This current is G-protein linked, susceptible to block by cetiedil and Ba^{2+} but is insensitive to UCL1880. It is worth bearing in mind that there are many G protein-modulated currents in cardiac tissue including an L-type Ca^{2+} current (Yatani *et al.*, 1987, 1988), the fast inward Na^+ current (Ono *et al.*, 1989, Matsuda *et al.*, 1992) and Cl^- currents (Ackerman and Clapham, 1993). Intracellular dialysis with GTP- γ -S could in principle influence any of these currents.

The recovery from block took increasingly longer at higher concentrations requiring more than 15 minutes after 2 μM UCL1880. This may be due to the time taken for the drug to leave the lipid of the cell membrane. In keeping with this, it was observed that the adenosine current 5 minutes after washout of high concentrations of UCL1880 still displayed increased fade (as illustrated for UCL1495 in Figure 3.3.1.3C and D) - use-dependence- suggesting that the drug molecules are still within the membrane in close proximity to the channel.

UCL1495 also appears to have a use-dependent action. Lower concentrations of UCL1495 cause an increase in the spontaneous decline in the response as compared with the control which is in contrast to the action of the parent compound cetiedil which displayed no use-dependence in inhibition of the adenosine current (Jones 1995). Interestingly, cetiedil appears to block the Ca^{2+} -activated- K^+ channels in red blood cells in a use-dependent fashion (Benton 1995).

Quantification of the use-dependent action of UCL1880 and UCL1495 indicated that it was more apparent with low concentrations of the drug. This may result from the more rapid onset of block by the higher concentrations which masks any use-dependence. It was observed that some of the adenosine responses did not show an obvious peak and the application of the blocker did

not then result in so large an acceleration in decay as compared with the more 'peaky' responses. This is probably because of a relatively slow perfusion system and the correspondingly slow development of the adenosine current in such experiments. UCL1880 and UCL1495 block the steady state phase more so than the peak current phase which is reflected in the IC_{50} values listed in Table 3.3.3. UCL1880 is more than twice as potent on the maintained phase than on the peak current phase. UCL1495 is also more effective on the maintained phase but only by ~ 1.5 times. The block by UCL1880 could in principle involve either a direct channel blocking action or an allosteric interaction with the channel protein in such a fashion as to favour the formation of an inactivated conformation of the channel. This could be further investigated by single channel recordings.

The results show that the structural analogue of clotrimazole, UCL1880, is a potent blocker of the G-protein-gated muscarinic K^+ channel. There are other known potent blockers of this current such as disopyramide which blocks the ACh-induced current in guinea-pig atrial cells with an IC_{50} of $1.5\mu M$ ($-53mV$) though this is probably mainly due to muscarinic receptor block since this compound was less effective in blocking the adenosine-activated or GTP- γ -S induced current (Nakajima *et al.*, 1989). In the same study quinidine was just as effective in blocking the GTP- γ -S supported current as the adenosine-induced current having an IC_{50} of $\sim 10\mu M$, suggesting that it blocks the functions of the activated G-proteins and/or the K^+ channel itself. Since quinidine is a well known blocker of other K^+ channels it is not surprising that it also blocks the K_{ACh} channels. An inhibitory action of quinidine was also seen in another study which looked at the mechanism by block of various anti-arrhythmic compounds (Inomata *et al.*, 1991). SUN1165 [N-(2, 6-dimethylphenyl)-8-pyrrolizidinylacetamide hydrochloride hemihydrate)] and disopyramide had IC_{50} values of $29\mu M$ and $1.7\mu M$, respectively, against the ACh response in guinea-pig atrial cells. However in GTP- γ -S loaded cells the IC_{50} values for both of these compounds were greater than $100\mu M$ and it was concluded that these compounds mainly block the muscarinic receptor (Inomata *et al.*, 1991). In the same study flecainide and quinidine had IC_{50} values of $3.6\mu M$ and $1.6\mu M$,

against ACh respectively, and against the GTP- γ -S the values were 5.3 μ M for flecainide and 4.4 μ M for quinidine. Thus flecainide and quinidine seem likely to act at the G-protein and/or the channel. The Ca^{2+} channel antagonist, verapamil, blocks the ACh current in guinea-pig atrial cells with an IC_{50} of 1 μ M and has also been shown to bind to muscarinic receptors. However the GTP- γ -S current was also blocked with an IC_{50} of 3 μ M, and it was concluded that verapamil affects the functions of the muscarinic ACh receptor as well as G proteins and/or K^{+} channel (Ito *et al.*, 1989).

UCL1880 is a much more potent inhibitor of the adenosine-induced current than any of the above mentioned compounds and clearly merits further investigation.

3.3.8.4.2: GLIBENCLAMIDE BLOCK OF THE ADENOSINE CURRENT.

Glibenclamide blocked the steady state adenosine current with an IC_{50} of $\sim 24 \mu\text{M}$ and was not significantly more effective in reducing the peak current (Figure 3.3.1.4 and 3.3.3.2C). The evidence from the GTP- γ -S experiments makes it unlikely that the adenosine receptors are blocked. Hence the action is more likely to be at the channels. However the exact mechanism of the block by glibenclamide is not clear, though the absence of use-dependence may mean that glibenclamide blocks the channels in their closed forms.

Glibenclamide is, of course, a well characterized blocker of ATP-sensitive channels where its action is known to be through the sulphonylurea receptor subunit. One possibility is that the glibenclamide sensitivity observed in the present work is a consequence of the activation of K_{ATP} channels by adenosine, as suggested by Li *et al.*, (1995). Glibenclamide is usually considered to be highly selective in its actions against K_{ATP} channels. However, Li *et al.*, (1995) showed that 20 μM glibenclamide blocked up to 70% of the adenosine-induced current in guinea-pig atrial cells but not the acetylcholine-induced current. In the same study adenosine was shown to activate two conductance levels in single channel recordings, only one of which was observed on application of acetylcholine. It was suggested that the additional openings

induced by adenosine were due to K_{ATP} channels. The concentration of glibenclamide used by Li *et al* was however much higher than usually needed to block K_{ATP} channels. Why adenosine but not ACh should activate K_{ATP} is not clear but it may be noted that in cat atrial myocytes it was found that ACh was able to activate a glibenclamide sensitive current and this was attributed to opening of K_{ATP} channels (Wang and Lipsius 1995). In ventricular myocytes of the neonatal rat the application of adenosine and intracellular GTP had the same effect as GTP- γ -S in restoring the K_{ATP} current blocked by intracellular ATP (Kirsch *et al.*, 1990) suggesting that the ATP channels may be coupled to adenosine receptors via G-proteins. A recent study in rat ventricular cells has shown that adenosine activates K_{ATP} channels via protein kinase C activation since the adenosine response is reduced in the presence of specific PKC inhibitors (Köppel and Standen 1997).

Although all these studies provide evidence for the activation of K_{ATP} channels by adenosine in cardiac myocytes, the interpretation of the action of glibenclamide still requires caution. Although low concentrations may produce a specific block of these channels, high concentrations - possibly even at 1-10 μ M - may exert other actions (Beech, 1997).

In the present work, the response to adenosine was found to be blocked by glibenclamide with an IC_{50} of 24 μ M. However the results to be described in Chapter Five show that the levcromakalim-activated current is 66% blocked by glibenclamide at 1 μ M, a much lower concentration. It has been reported that the block by glibenclamide of the K_{ATP} channel in cardiac myocytes occurs with an IC_{50} of between 5-20 μ M (Nichols and Lederer, 1991). As K_{ATP} is also thought to be activated by $G\alpha$ -subunits (Kirsch *et al.*, 1990, Ito *et al.*, 1992) the presence of GTP- γ -S in the patch pipette in the experiments summarized in Figure 3.3.6.4 may have activated some K_{ATP} current which would have been expected to be blocked by 1 μ M glibenclamide. Yet 1 μ M glibenclamide blocked that current by only 12% and raising the concentration to 10 μ M increased the block to only ~40%. Thus if there is any activation of $I_{K_{ATP}}$ by adenosine in these neonatal rat atrial myocytes it is rather small.

The actions of glibenclamide and other sulphonylureas result from binding at the SUR. Could there be a SUR associating with the K_{ACh} channel protein? My results show that glibenclamide blocks the adenosine current and the GTP- γ -S current to the same degree so that receptor block is unlikely. The blocks of the peak current and steady state current phases are not significantly different and there is no evidence either for a use-dependent action of glibenclamide or for any voltage-dependent effect thus effectively arguing against a channel block (although it is possible that the lack of voltage-dependence may reflect binding of the molecule outside the electrical field of the membrane). It can also be speculated that the channels may not be the only target for the glibenclamide bound SUR and that this complex may interfere with the G- protein or its activation of the channel. The binding of the SUR to inward rectifier K^+ channels other than the K_{ATP} channel is discussed further in Chapter Six.

3.3.8.4.3: BLOCK OF THE ADENOSINE CURRENT BY Ba^{2+} .

The known K^+ channel blocker Ba^{2+} blocked the adenosine current in a voltage- and concentration-dependent manner. The results in Figure 3.3.7.3 show that Ba^{2+} is more effective in blocking the current at -120mV than at -50mV; the IC_{50} value is $3.8\mu M$ at -120mV and $34.9\mu M$ at -50mV. This has also been observed in guinea-pig atrial cells where the block of the adenosine-induced current occurs with an IC_{50} of $125\mu M$ at 0mV and $15.2\mu M$ at -80mV (Zang *et al.*, 1995). Other studies have shown that the block of $I_{K_{ACh}}$ by Ba^{2+} is dependent upon the membrane potential and on the K^+ concentration bathing the cell. This evidence supports the notion that Ba^{2+} blocks the channel preferentially when it is in the open state (Zang *et al.*, 1995). The dependence of the IC_{50} values on the membrane potential is thought to be due to Ba^{2+} binding to a site within the pore of the channel and within the electrical field of the membrane. The block is described in most studies as 1:1 binding of Ba^{2+} to a binding site in the channel since in most studies the Hill coefficient for the inhibition curve is ~ 1 (Standen *et al.*, 1978, Zang *et al.*, 1995, Carmeliet and Mubagwa, 1996). The Hill coefficients in this study are significantly lower

being 0.6 ± 0.14 for the block at -50mV and 0.5 ± 0.13 at -120mV . The reason for the difference between the values in this study and published values remains unclear. A value of less than 1 may indicate that there are two components of the activated current which are blocked with different affinities. This is further discussed in Chapter Six.

The voltage-dependence of the Ba^{2+} block of the current was compared with the effect of voltage on the block produced by the other compounds since Ba^{2+} is a well established open-channel blocker. Table 3.3.4 shows no voltage-dependence of the compounds investigated in this study. This does not completely rule out a voltage-dependent block as it may be possible that the molecules block in their uncharged form, or at the open channel outside the voltage field of the membrane. Alternatively this result could be explained by the fact that at relatively high concentrations of blocker used to investigate the effect of voltage there is already a substantial block and an increased block due to the voltage change may not be distinguishable. In retrospect it would have been informative to use a lower concentration (such as the IC_{50} value) to compare the block at the different potentials. Nevertheless the evidence from the effect of the blockers on the ratio of peak to steady state current does suggest a use-dependent effect. UCL1880 and UCL1495 may block the channel in their non-ionized form as has been shown in skeletal muscle and *Helix* neurons for the blocking actions of external 4-aminopyridine which increases with alkaline pH (Gillespie, 1977, Plant and Standen 1982). This has been explained by the increased proportion of the 4-AP in its uncharged lipid-soluble form as the pH approaches its pK_a of 9.17. The pK_a values for UCL1880 and UCL1495 have not yet been determined but are needed to provide information as to what percentage of the drugs are in their ionized or non-ionized forms at the pH tested in this study.

The evidence thus far suggests that the actions of UCL1880 and UCL1495 occur at the channel. To further confirm this the compounds were tested on two other inward rectifiers found in cardiac myocytes, I_{K_1} and $\text{I}_{\text{K}_{\text{ATP}}}$. It would also be interesting to see if any specificity was displayed by any of the

compounds. In the following chapter, results of the action of UCL1880, UCL1495, glibenclamide and Ba^{2+} on I_{K_1} are presented.

CHAPTER FOUR: THE BACKGROUND INWARD
RECTIFIER POTASSIUM CHANNEL IN NEONATAL RAT
ATRIAL MYOCYTES.

CHAPTER FOUR: THE BACKGROUND INWARD RECTIFIER POTASSIUM CHANNEL IN NEONATAL RAT ATRIAL MYOCYTES.

4.1: INTRODUCTION:

Katz (1949) first described the classical inward rectifier, I_{K_1} , in frog skeletal muscle. It is a 'strong' rectifier with little outward current flow at potentials positive to E_K . Its electrophysiological properties - such as more current flowing in the inward direction and the dependence of the kinetics of the channel on the extracellular K^+ concentration - are such that I_{K_1} has a role in maintaining the resting membrane potential and in regulating the action potential duration (in conjunction with voltage-gated K^+ channels) thereby controlling electrical excitability.

The I_{K_1} channel has been described in many tissues and has been studied extensively in frog skeletal muscle (Standen & Stanfield 1978, 1979), starfish egg (Hagiwara *et al.*, 1978) and tunicate eggs (Ohmori *et al.*, 1978). These channels have several characteristic features such as block by intracellular Mg^{2+} and polyamines, as well as by extracellular Ba^{2+} and other cations. Removal of intracellular Mg^{2+} abolishes the rectification leading to ohmic conductance in isolated patches (Matsuda *et al.*, 1987; Vandenberg 1987; Martin *et al.*, 1995). The block by intracellular Mg^{2+} shows the characteristics of open channel block such as interruptions of single channel events which increase with high concentrations of Mg^{2+} , and voltage-dependence (Vandenberg, 1987). The rectification occurring at potentials positive to E_K is thought to be due not only to Mg^{2+} but also to the voltage-dependent block by intracellular polyamines such as spermine and spermidine (Bechem *et al.*, 1983; Matsuda *et al.*, 1987; Shioya *et al.*, 1993; Lopatin *et al.*, 1994; Martin *et al.*, 1995). As already discussed in Chapter 1, specific amino acid residues within the channel structure are now known to influence the extent of rectification (Faklar *et al.*, 1994; Lu and Mackinnon, 1994; Pessia *et al.*, 1995).

The inwardly rectifying conductance (I_{K_1}) in cardiac myocytes is the dominant component of the resting conductance in ventricular (Ber Heler & Reuter 1970) and atrial tissue (Kurachi 1985). The inward rectifier helps to keep

the membrane potential close to E_K during diastole and it ensures that at plateau potentials there is little outward current so that K^+ efflux is reduced during this phase of the action potential. The reduction in K^+ conductance helps to maintain the plateau which is important for Ca^{2+} entry and consequently the pumping action of the heart. Repolarisation eventually occurs due to activation of the delayed rectifier current and inactivation of Ca^{2+} current. As repolarisation proceeds, the current through I_{K_1} increases which accelerates the return to the diastolic potential.

Thus the I_{K_1} channel plays a role in determining the shape of the cardiac action potential by:

- (1) setting the diastolic membrane potential
- (2) maintaining the plateau phase
- (3) contributing to rapid repolarisation

4.2: AIMS:

The aim of the work described in this chapter was to investigate the actions of UCL1880, UCL1495, glibenclamide and Ba^{2+} on the background current in rat atrial myocytes for subsequent comparison with their actions on the G-protein coupled potassium channel and the levcromakalim-activated potassium channel. Ba^{2+} was tested for comparative purposes as it is a well-characterised blocker of the channel.

4.3: RESULTS

4.3.1: THE I_{K_1} CURRENT

The current through I_{K_1} channels was activated in response to voltage steps from a holding potential of -50mV to between -120mV and 0mV in +10mV increments. Each voltage step lasted 500ms. Full steady state current-voltage relationships were plotted to demonstrate the inwardly rectifying nature of these currents. Figure 4.3.1.1.A shows a representative whole cell voltage clamp experiment. Depolarising steps positive to -40mV resulted in an inward

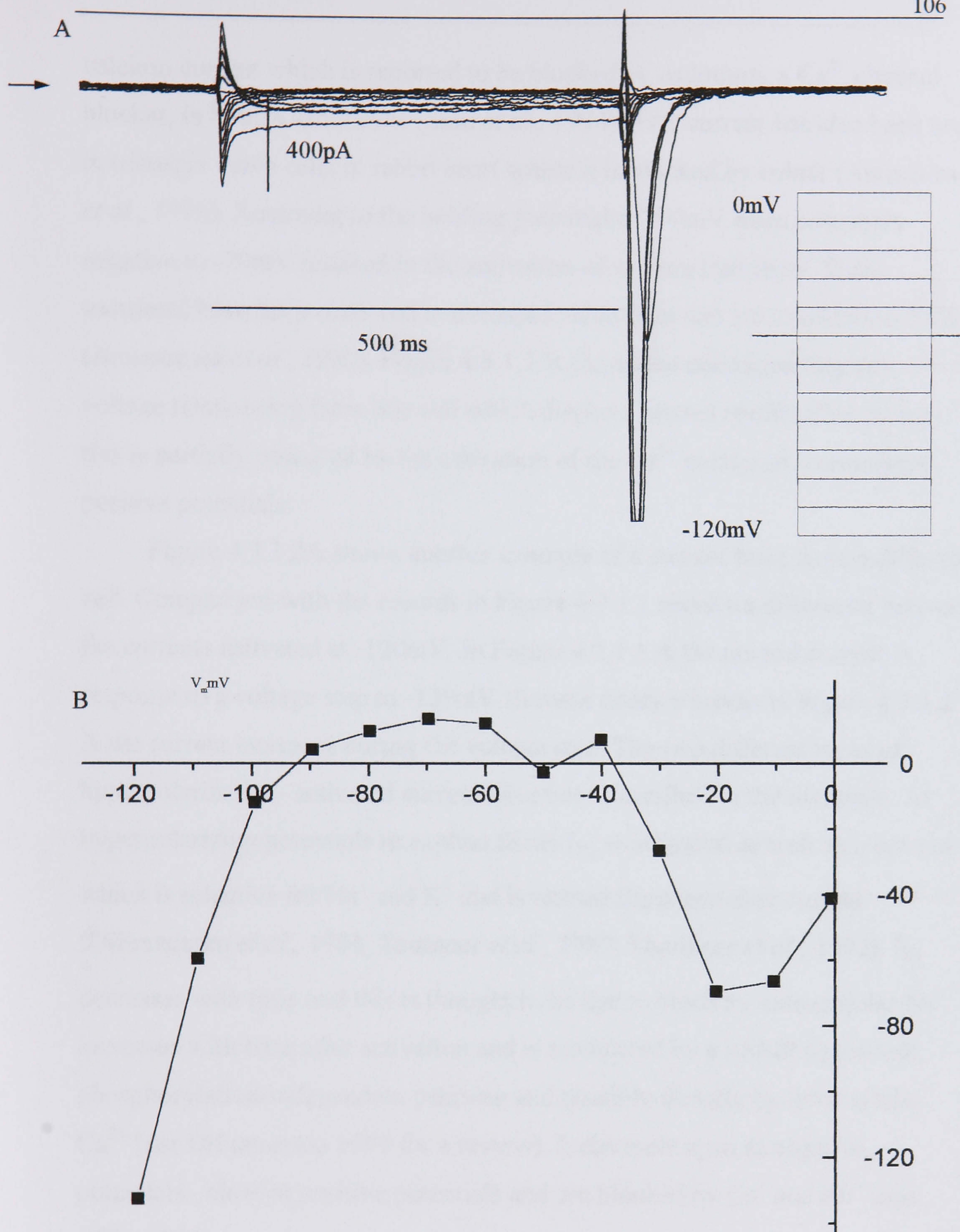


Figure 4.3.1.1. A shows the current traces in response to voltage steps between -120 mV and 0 mV, in +10 mV increments from a holding potential of -50 mV. Each step lasted 500 ms. The arrow on the left indicates the zero current level. The voltage protocol is also shown. B displays the current-voltage relationship for the same cell, plotted from the currents observed just before the end of the step.

calcium current which is reported to be blocked by cadmium, a Ca^{2+} channel blocker, in human atrial cells (Sato *et al.*, 1994). This current has also been seen in tricuspid valve cells in rabbit heart where it is blocked by cobalt (Anumonwo *et al.*, 1990). Returning to the holding potential of -50mV from potentials negative to -70mV resulted in the activation of sodium transients. Such transients have been observed in tricuspid valve cells and are sensitive to TTX (Anumonwo *et al.*, 1990). Figure 4.3.1.1 B shows the corresponding current-voltage relationship from this cell which displays inward rectification though this is partially obscured by the activation of the Ca^{2+} current at increasingly positive potentials.

Figure 4.3.1.2A shows another example of a current trace from a different cell. Comparison with the records in Figure 4.3.1.1 reveals a difference between the currents activated at -120mV. In Figure 4.3.1.1 A the inward current in response to a voltage step to -120mV shows a decay whereas in Figure 4.3.1.2 A the current increases during the voltage step. The two different types of hyperpolarization-activated current have been described in the literature. At hyperpolarizing potentials in cardiac tissue I_{K_1} is activated as well as I_f current which is selective for Na^+ and K^+ and is termed the pacemaker current (DiFrancesco *et al.*, 1984, Tourneur *et al.*, 1987, Thuringer *et al.*, 1992). I_{K_1} decreases with time and this is thought to be due to block by extracellular Na^+ . I_f increases with time after activation and is modulated by a cAMP dependent, phosphorylation-independent pathway and possibly directly by intracellular Ca^{2+} (see DiFrancesco 1993 for a review). I_f channels open at negative potentials, close at positive potentials and are blocked by Cs^+ and Rb^+ ions (Hille 1991).

The current at -120mV in Figure 4.3.1.1A decreases with time and is therefore mainly I_{K_1} whereas the current shown in Figure 4.3.1.2A increases with time and is most likely a mixture of I_{K_1} and I_f . In the atrial cells used in this investigation the majority of currents observed were predominantly I_{K_1} .

Figure 4.3.1.2 B shows the current-voltage relationship for all of the current traces displayed in 4.3.1.2.A. A substantial amount of inward current is

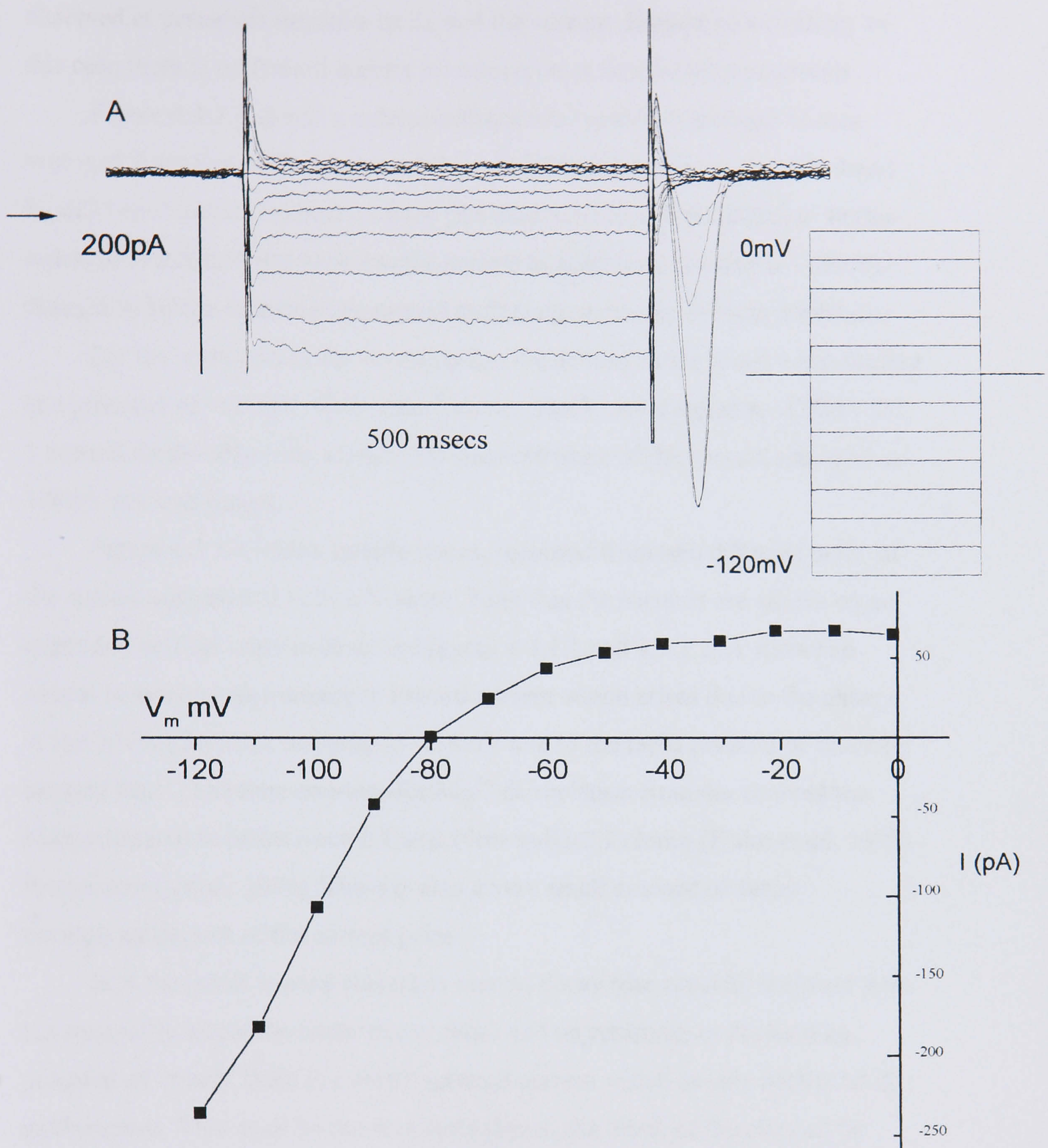


Figure 4.3.1.2.A shows current traces in response to voltage steps between -120mV and 0mV. Steps were 500ms in duration and were applied in +10mV increments. The arrow on the left indicates the zero current level. B illustrates the I-V plot for the steady state current in A. The voltage protocol is shown in the inset.

observed at potentials negative to E_K and the current displays rectification. In this case there is no inward current switching on at depolarising potentials.

Figure 4.3.1.3 shows a current-voltage relationship composed of data averaged from five cells. The reversal potential is quite close to the calculated E_K of -94mV and the current-voltage plot displays inward rectification. In this averaged response there is an inward current at potentials positive to -40mV, thought to be due to a calcium current switching on at depolarising potentials.

For the main part of the investigation the actions of the drugs were studied at a potential of -120mV. Cells were held at -50mV and stepped to -120mV for 1 second. In the following section the characteristics of the current activated at -120mV are considered.

Figure 4.3.1.4 shows sample traces, recorded from two different cells, of the current activated at -120 mV alone. Note that the currents are shown on an expanded vertical scale to those in Figures 4.3.1.1 and 4.3.1.2. A shows an almost instantaneous increase in inward current which arises due to the change in the driving force on stepping to -120mV and to the rapid reversal of block by internal Mg^{2+} . The time constant for Mg^{2+} dissociation from the channel has been estimated to be between 0.1 and 10ms in Kir2.1 clones (Kubo *et al.*, 1993, Ruppersberg *et al.*, 1996). There is also a very small amount of decay throughout the rest of the current pulse.

In B the initial inward current is seen to decay (the capacity transient does not materially affect the initial decay seen) and on returning to the holding potential of -50mV there is a small outward current which decays within 10-20 milliseconds. This may be due to a reduction in the block of the channel by intracellular Mg^{2+} at the hyperpolarised potentials which is then re-established on returning to -50mV (Kelly *et al.*, 1992).

The decay seen in B is typical of the background I_{K1} current seen at potentials negative to E_K in many different tissues (Biermens *et al.*, 1987; Harvey and Ten Eick 1989; Kelley *et al.*, 1992; Dominique-Ashen *et al.*, 1995) and is attributed to external block by monovalent cations, in particular Na^+ . It has been shown that the removal of Na^+ from the external solution reduces this decay and that this Na^+ -dependent inactivation is voltage-dependent, becoming

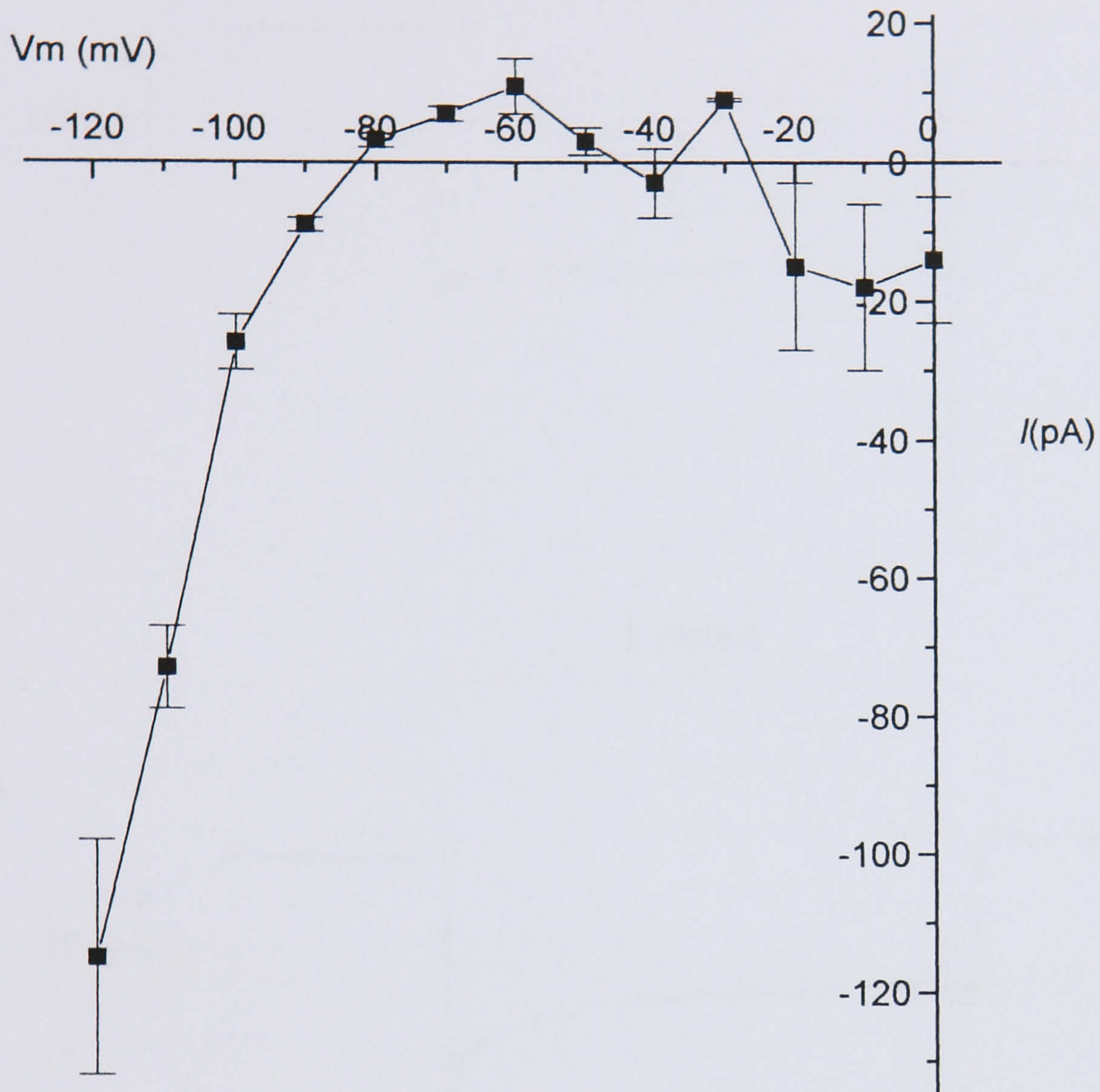


Figure 4.3.1.3: Current-voltage relationship for the background inwardly rectifying potassium channel in rat atrial myocytes. Data are averages from 5 cells. Cells were held at -50mV and stepped from between -120 and 0mV in +10mV increments. Each step lasted 1 second. Currents were measured towards the end of the 1 second pulse.

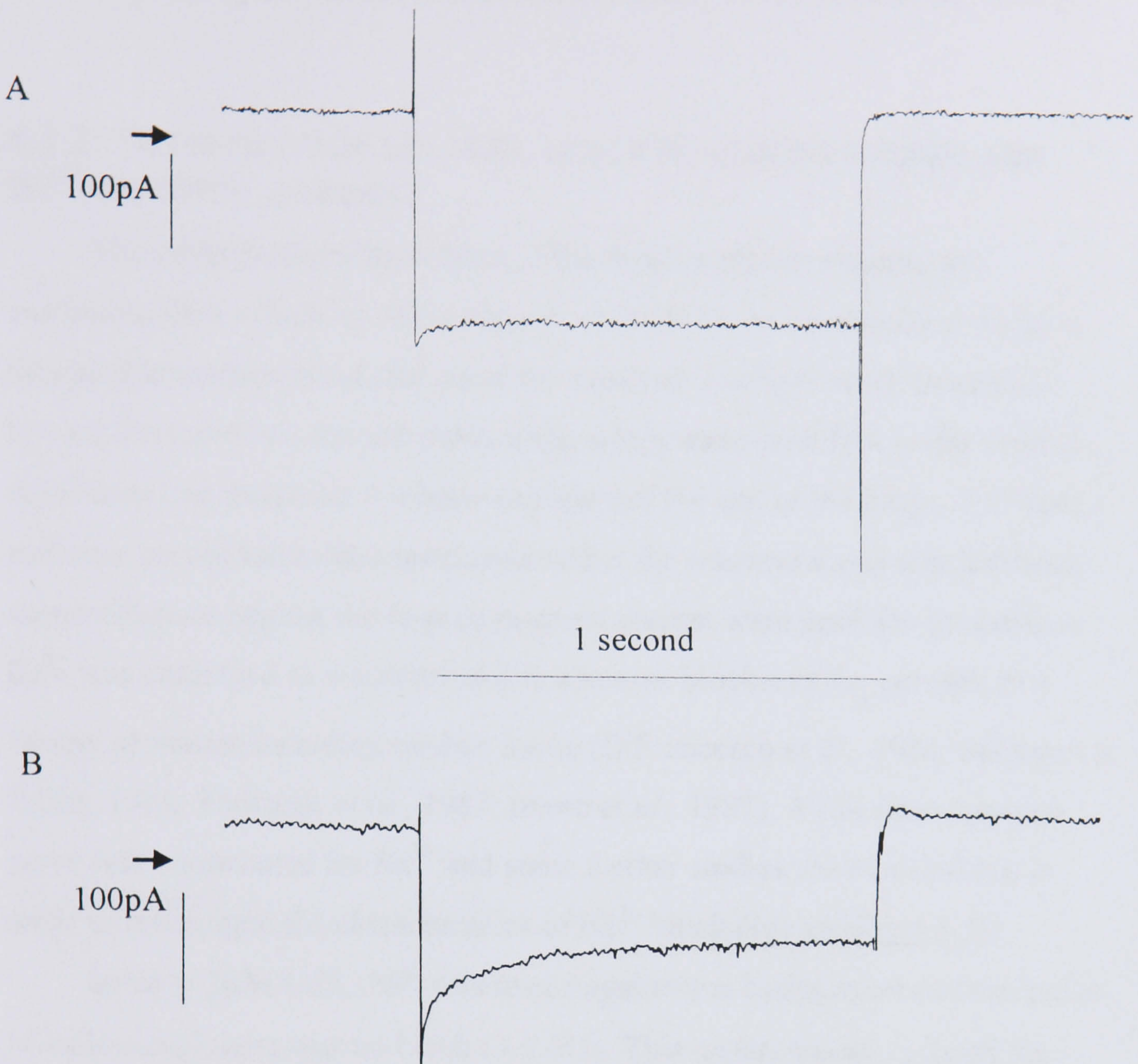


Figure 4.3.1.4: Two examples of the current activated at -120mV from a holding potential of -50mV . Traces are from different atrial myocytes. Each example is an average of 10 traces. The steps were applied when the cell was bathed with Krebs solution. The time scale is the same in each example. Each step was 1 second in duration.

more marked at potentials negative to $\sim -120\text{mV}$ (Ohmori, 1978; Biermans *et al.*, 1987; Harvey & Ten Eick, 1989; Kelley *et al.*, 1992; Voets *et al.*, 1996).

4.3.2: THE EFFECTS OF UCL1880, UCL1495, GLIBENCLAMIDE AND Ba^{2+} ON THE I_{K_1} CURRENT.

The possible blocking actions of the drugs were investigated by examining their effects on the current at -120mV i.e. at a potential at which a measurable amount of current could be observed. Currents were measured toward the end of a 1 second pulse at the steady state level first under control conditions and then after 4 minute exposure of the cell to the drugs. Full dose - response curves were not constructed, rather the concentrations that had been found effective against the G-protein-gated current were used for comparison. Ba^{2+} was employed as a control as it is a known blocker of I_{K_1} currents in a variety of tissues including cardiac tissue (DiFrancesco *et al.*, 1984; Sakmaan & Trube, 1984; Tourneur *et al.*, 1987; Imoto *et al.*, 1987). A full dose response curve was constructed for Ba^{2+} and some further studies were carried out in order to investigate the characteristics of Ba^{2+} block (see section 4.3.3).

Initially $2\mu\text{M}$ UCL1880 was tested against the background current and in 11 cells tested there was no block ($3 \pm 8\%$). This concentration reduced the response to adenosine by $\sim 90\%$ (Chapter 3). Increasing the concentration of UCL1880 to $10\mu\text{M}$ resulted in a block of $22 \pm 11\%$ ($n=11$). Figure 4.3.2.1 shows the effect of $10\mu\text{M}$ UCL1880 and of $10\mu\text{M}$ Ba^{2+} on the background current. A shows the control current which displays a small amount of decay after the initial inward current. B shows the effect of Ba^{2+} . It can be seen that Ba^{2+} had only a small effect on the instantaneous inward current. Subsequently the current declines within 50ms to approximately 60% of the control steady state level. C shows the effect of $10\mu\text{M}$ UCL1880. The block observed is different from the Ba^{2+} block in that the total inward current has been reduced and there is no evidence of a subsequent decay, as observed with Ba^{2+} .

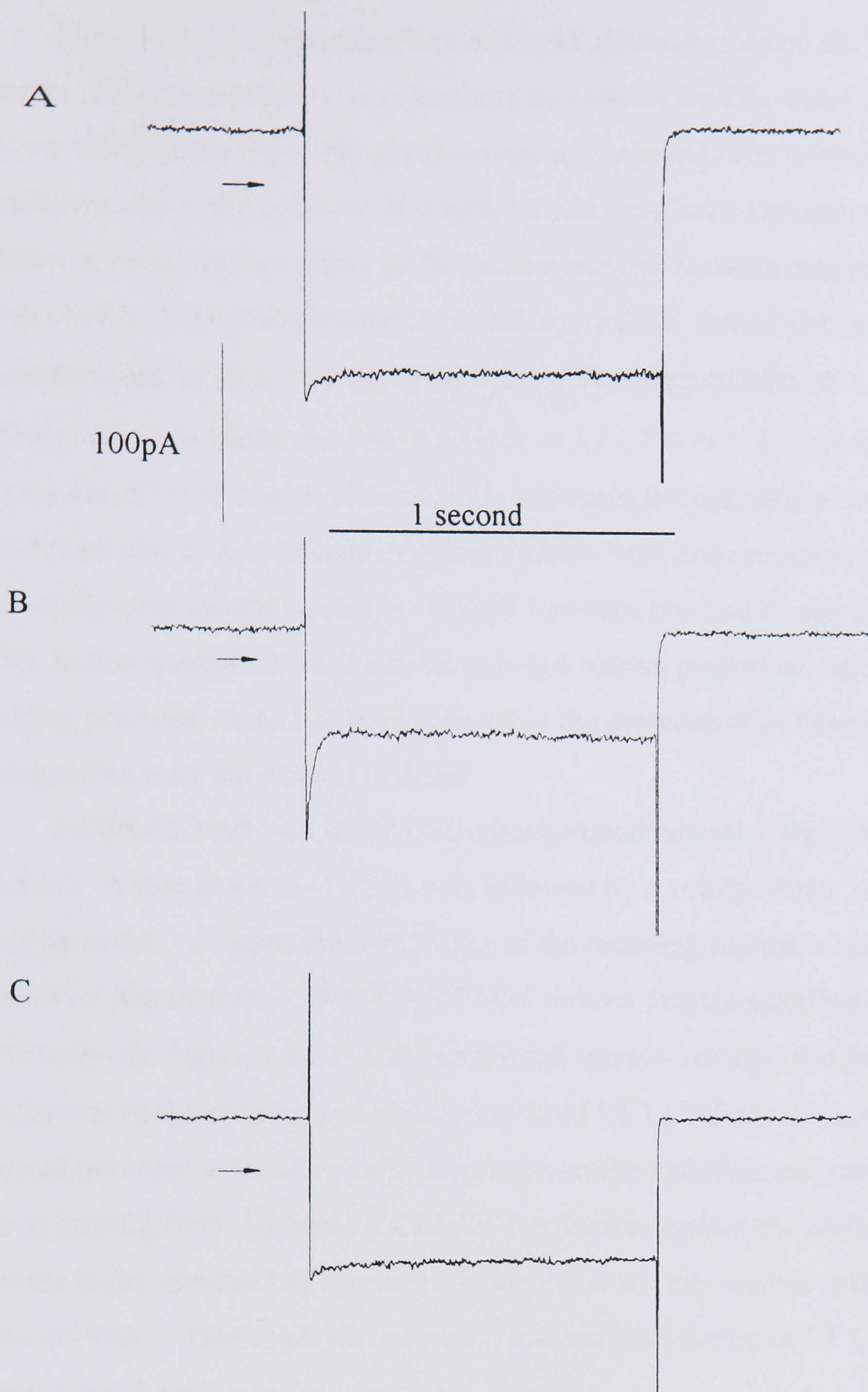


Figure 4.3.2.1: The effect of $10\mu\text{M Ba}^{2+}$ and $10\mu\text{M UCL1880}$ on the background inward rectifier current in a neonatal rat atrial myocyte. The current is shown under control condition, A, after 4 minute incubation with $10\mu\text{M}$ barium, B, and after 4 minute incubation with $10\mu\text{M UCL1880}$, C. Trace C was taken ~ 5 minutes after Trace B. The effect of Ba^{2+} was readily reversible. In this experiment the block caused by UCL1880 was larger than usual. The cell was stepped down to -120mV for 1 second from a holding potential of -50mV . All drugs were bath applied. The scale bars for B and C are as in A.

Figure 4.3.2.2 shows the effect of 20 μ M glibenclamide on the background current. This concentration was chosen to be close to the IC₅₀ value determined for the block of the G-protein-gated current in Chapter 3. The currents in control conditions and in the presence of glibenclamide have been superimposed and there was no significant effect on the resting current. In this example any decay is so small and fast that the capacity transient masks it. 20 μ M glibenclamide caused a block of $12 \pm 13\%$ (n=5). Increasing the concentration of glibenclamide to 50 μ M resulted in a block of $13 \pm 8\%$ (n = 4). Figure 4.3.2.2B shows the effect of 50 μ M glibenclamide and again the currents in control conditions and in the presence of glibenclamide have been superimposed. Here the steady state inward current at -120mV has been blocked by approximately 30%. In this example there is also an outward current present on returning to the holding potential which has been reduced in the presence of glibenclamide. The tail currents were not studied in detail.

2 μ M UCL1495 was tested on the background current using a different protocol. A step down to -120mV was followed by a voltage ramp to 0mV, over 2s. Figure 4.3.2.3 shows the first 750ms of the resulting current in response to the initial step down to -120mV. UCL1495 did not cause a significant block ($5 \pm 13\%$, n= 5). Figure 4.3.2.4 A shows the full current-voltage relationship for the current in the absence and presence of 2 μ M UCL1495 for the same cell. The current in response to the voltage ramp is inwardly rectifying and there is no block by UCL1495. However UCL1495 is effective against the adenosine current in the same cell as shown in Figure 4.3.2.4B: this current is blocked by 65% at -50mV. This result shows clearly the selective action of UCL1495 against the G-protein gated current.

The effect of voltage on the block of the background current was also investigated. Figure 4.3.2.5 shows current-voltage relationships in the absence and presence of drugs. A shows the lack of effect of 2 μ M UCL1880 at any voltage. B is from a different cell and it can be seen that 2 μ M UCL1495 and 20 μ M glibenclamide did not have a great effect on the current at -120mV or at any other voltage. In contrast, it can be seen that 100 μ M Ba²⁺ blocked the current at potentials both negative and positive to E_K. Since these drugs had no

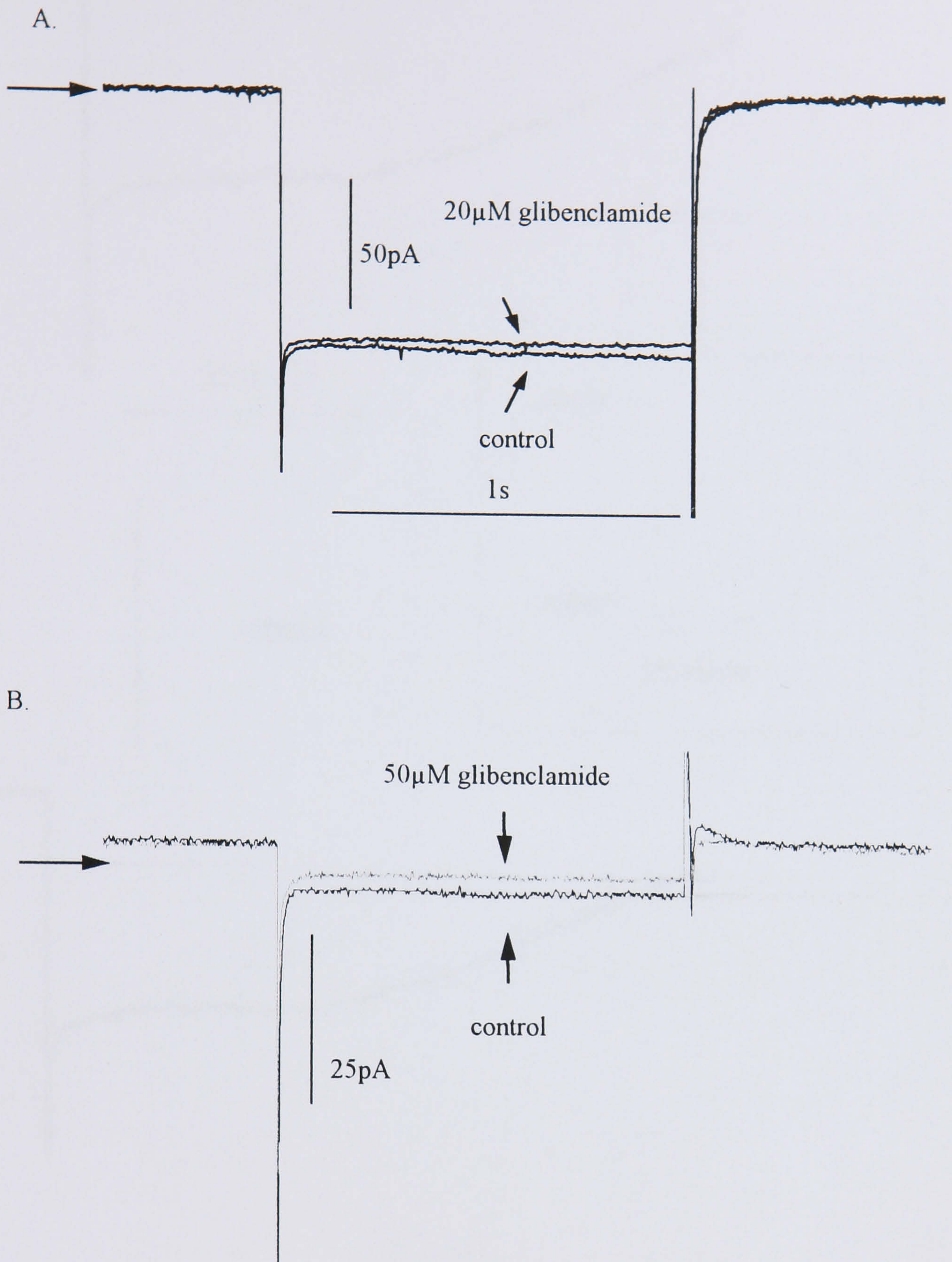


Figure 4.3.2.2: A shows the effect of 20 μ M glibenclamide on the background current at -120mV. B shows the effect of 50 μ M glibenclamide on a different cell. Traces are averages of 10. Each step is 1 second in duration. Arrows on the left indicate the zero current level. Glibenclamide was bath applied for 4 minutes. Cells were held at -50mV.

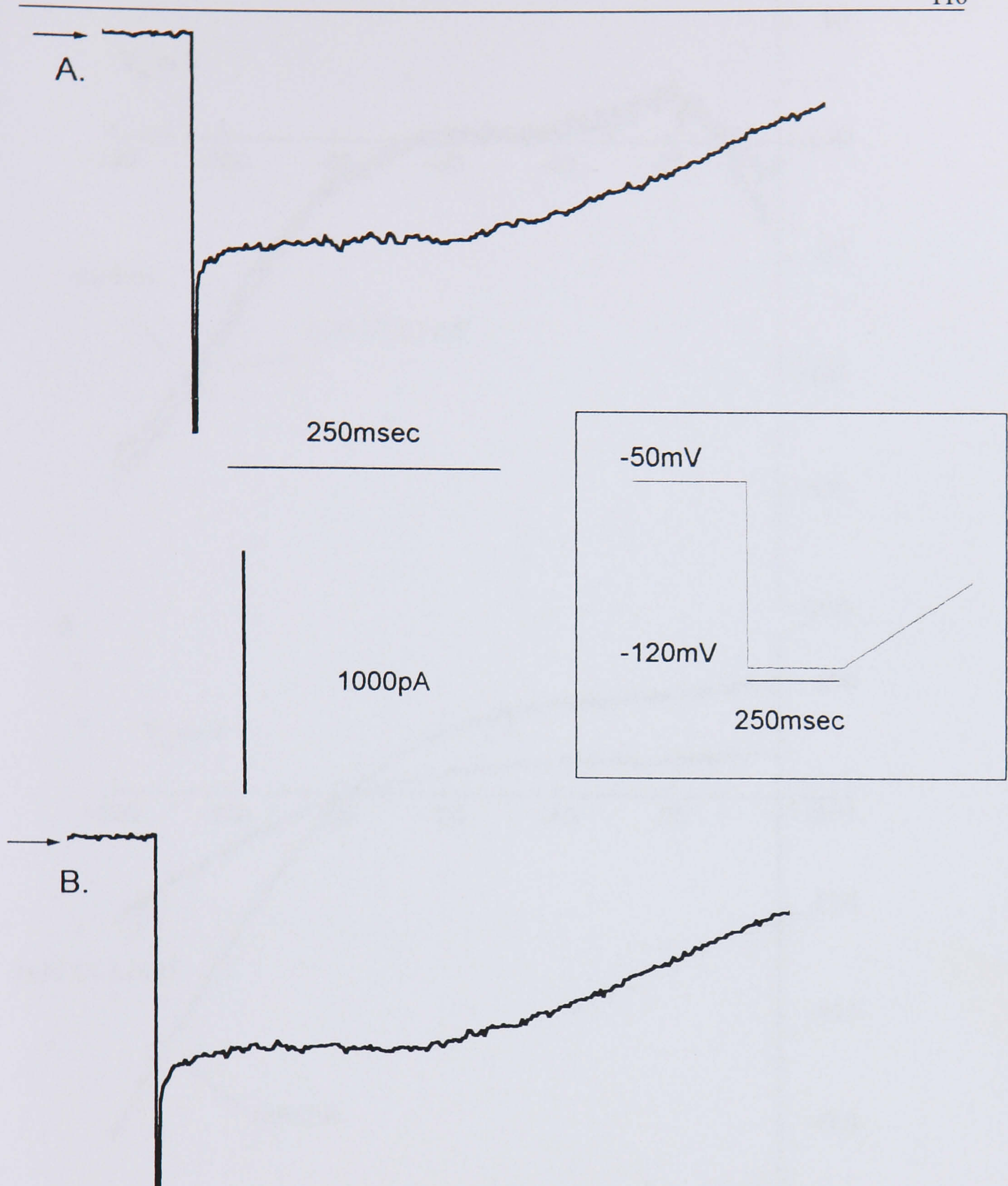


Figure 4.3.2.3: A The current in response to a voltage ramp as indicated inset. This is under control conditions. The cell was held at -50mV and stepped to -120mV for 250msecs. The whole of the trace is not shown here. B The effect of 2 μ M UCL1495. The cell was bathed in UCL1495 for 4 minutes before the voltage protocol was applied.

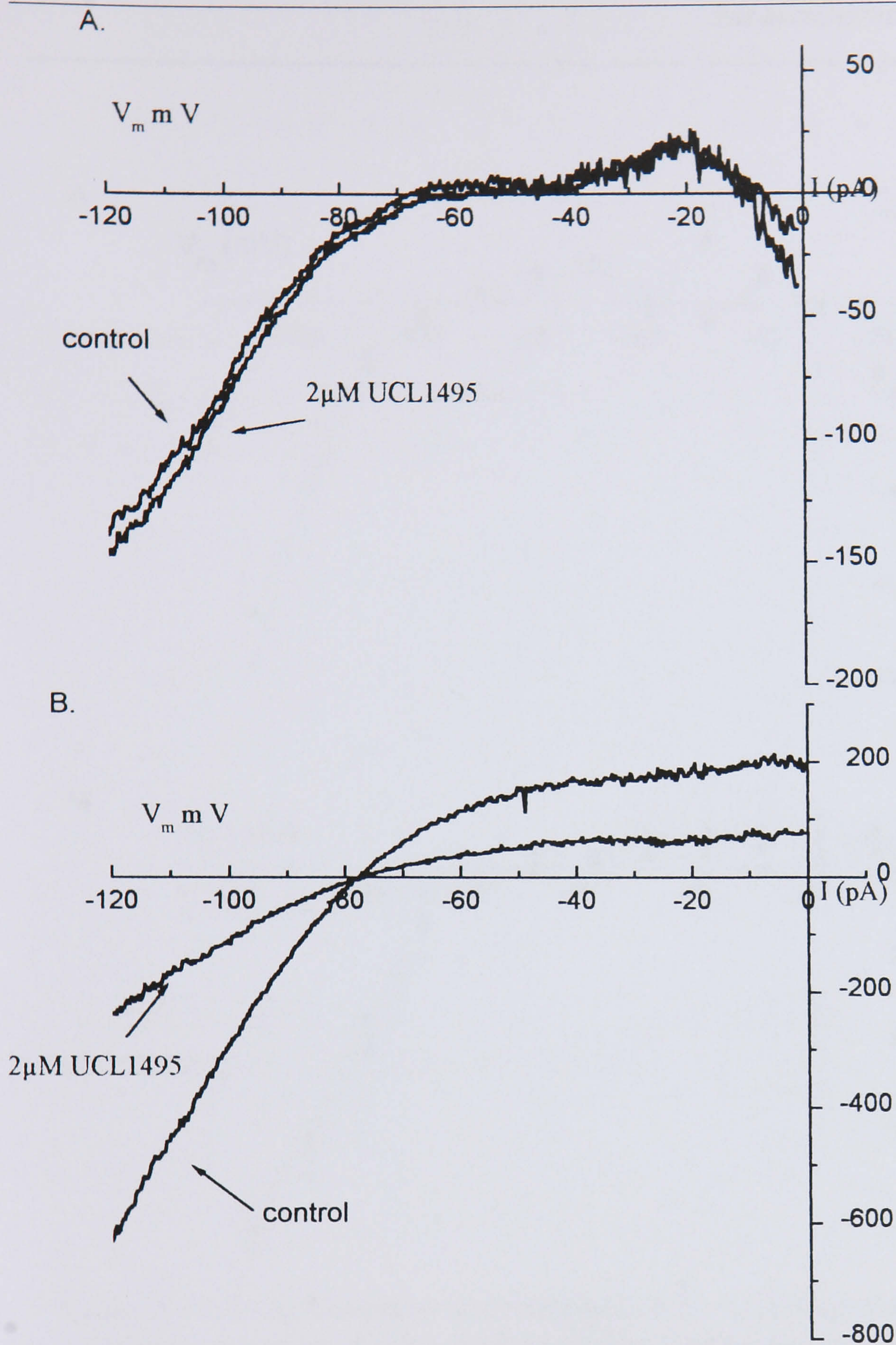


Figure 4.3.2.4: Current-voltage (IV) relationships showing the effect of $2\mu\text{M}$ UCL1495 on A, the background current and B, adenosine-induced current after subtracting the background current. Data are from the same cell which was held at -50mV and ramped from between -120 and 0 mV . Cells were incubated in UCL1495 for 4 minutes prior to ramping. All drugs were bath applied.

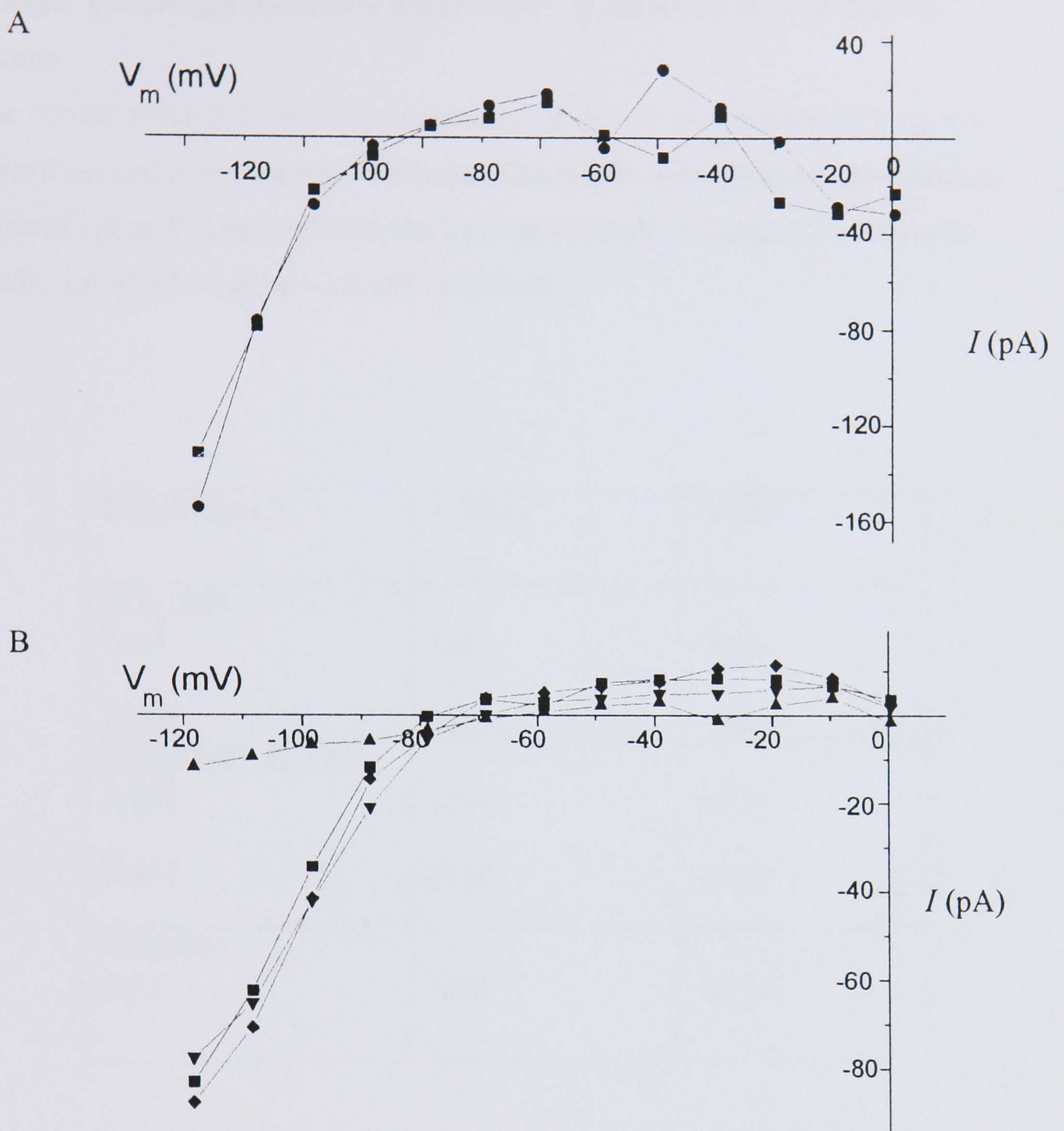


Figure 4.3.2.5 : A; Current-voltage relationship for the background current in rat atrial myocytes. Cells were held at -50mV and stepped from between -120mV and 0mV in $+10\text{mV}$ increments for 1 second duration. This was done for control ($-\blacksquare-$) and after 4 minutes incubation in $2\mu\text{M}$ UCL1880 ($-\bullet-$). B; data from a different cell showing the I-V plots for control, ($-\blacksquare-$) and in the presence of $2\mu\text{M}$ UCL1495, ($-\blacklozenge-$) $20\mu\text{M}$ glibenclamide ($-\blacktriangledown-$) and $100\mu\text{M}$ barium ($-\blacktriangle-$).

effect at -120mV it is not surprising that there was no effect at any other voltage. The voltage-dependent effect of Ba²⁺ is described in the following section.

The current present at the holding potential of -50mV was measured in control conditions and in the presence of drugs. The results from these experiments are summarised and compared with the block at -120mV in Table 4.3.1. There is hardly any block at either -120mV or -50mV.

COMPOUND	-120mV	-50mV
UCL1880 2µM	3±8	-2±11
10µM	22±11	-18±12
GLIBENCLAMIDE 20µM	12±13	10±11
50µM	13±8	-16±4
UCL1495 2µM	5±13	12±24

Table 4.3.1 The effect of voltage on the blocking action of the compounds on the background inward rectifier. Inhibitions were calculated at -120mV and -50mV.

A full dose response curve was constructed only for Ba²⁺ and is shown in Figure 4.3.2.6 together with the results from the above studies. The Ba²⁺ data were fitted with the Hill equation which, on constraining the maximum inhibition to 100%, gave an IC₅₀ value of 16.8±13µM and a Hill slope of 0.28 ± 0.10.

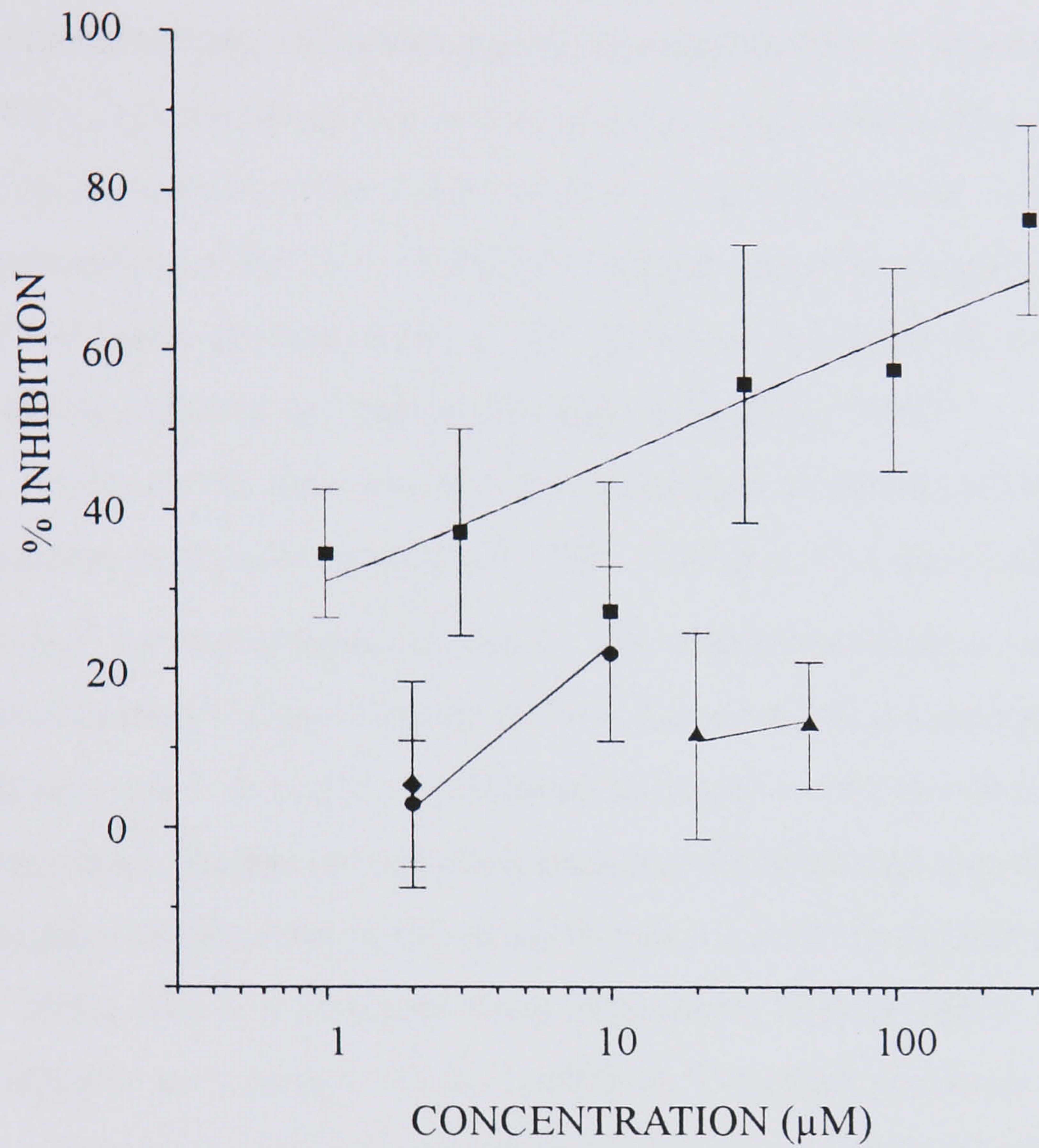


Figure 4.3.2.6: Log concentration-response curve for the effect of barium (squares), UCL1880 (circles), glibenclamide (triangles) and UCL1495 (diamonds) on the background current in neonatal rat atrial myocytes. A full relationship was constructed for barium only giving an IC_{50} value of $16.8\mu\text{M}$. Inhibitions were calculated at -120mV .

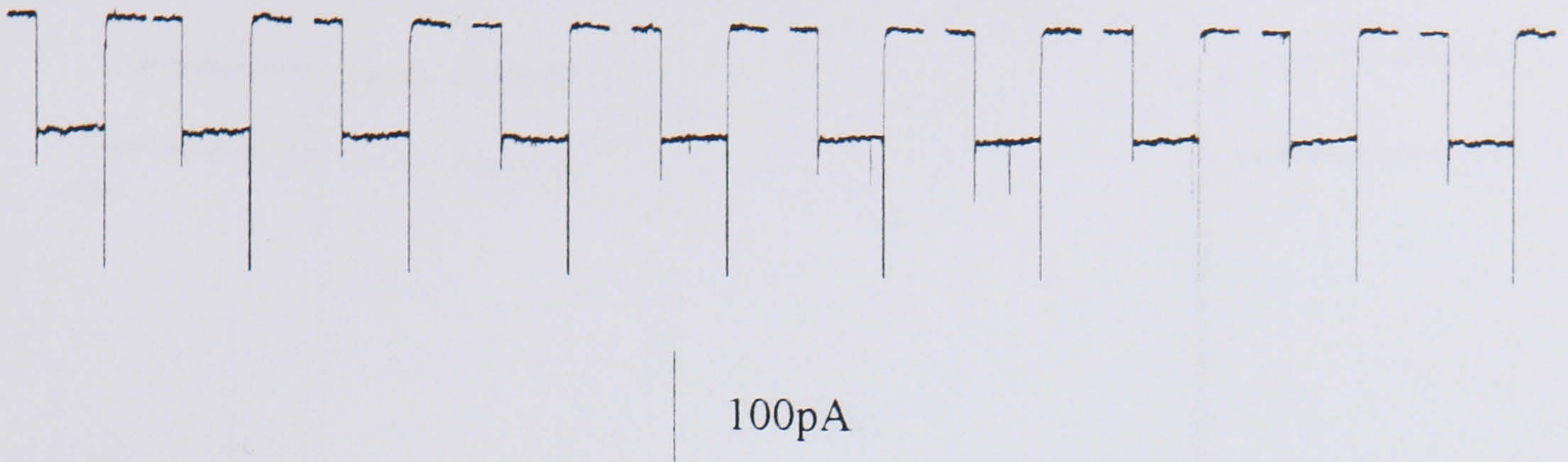
4.3.3: FURTHER INVESTIGATION OF THE Ba^{2+} BLOCK OF THE I_{K_1} CURRENT.

The action of Ba^{2+} on the background inward rectifier potassium channel has been widely examined in many tissues including the heart (Isenberg *et al.*, 1976, Hiraoka *et al.*, 1980) starfish egg (Hagiwara *et al.*, 1976, 1978) tunicate egg (Ohmori *et al.*, 1978) and skeletal muscle (Standen & Stanfield, 1978). Ba^{2+} has been shown to block the current in a time- and voltage-dependent manner (DiFrancesco *et al.*, 1984; Tourneur *et al.*, 1987; Imoto *et al.*, 1987) and at low concentrations of Ba^{2+} (5 - 50 μ M) the voltage- and time-dependent block is clear. At higher concentrations of Ba^{2+} the block is too fast for the time dependent nature to be obvious (Ishihara & Hiraoka, 1994).

Experiments were carried out to investigate the nature of the block of the I_{K_1} current in rat atrial myocytes by Ba^{2+} . Figure 4.3.3.1 shows the effect of 3 μ M Ba^{2+} on the background current. The current traces are in response to 10 hyperpolarising voltage steps to -120mV from a holding potential of -50mV. Each step was 1 second in duration and the steps were separated by 1 second. The top trace, A, shows control currents and it can be seen that there is decay throughout the duration of the pulse. Figure 4.3.3.1B shows the onset of the Ba^{2+} effect. The current traces are in response to 10 steps which were applied on immediately switching to the Ba^{2+} solution. The effect comes on at the sixth step and the current in each step thereafter decays more than in the control. C shows the currents after a 4-minute incubation period and it can be observed that a steady state has been reached which is at a lower level than in the control traces.

Figure 4.3.3.2 shows the averages of traces taken from Figure 4.3.3.1A and C. In the control there is observed an initial decay which occurs within a few milliseconds and subsequently the current remains quite steady for the remainder of the pulse duration. In the presence of 3 μ M Ba^{2+} the current decays to a new lower level and thereafter declines only slightly. Note that the outward holding current has also decreased in the presence of Ba^{2+} .

A



B



C

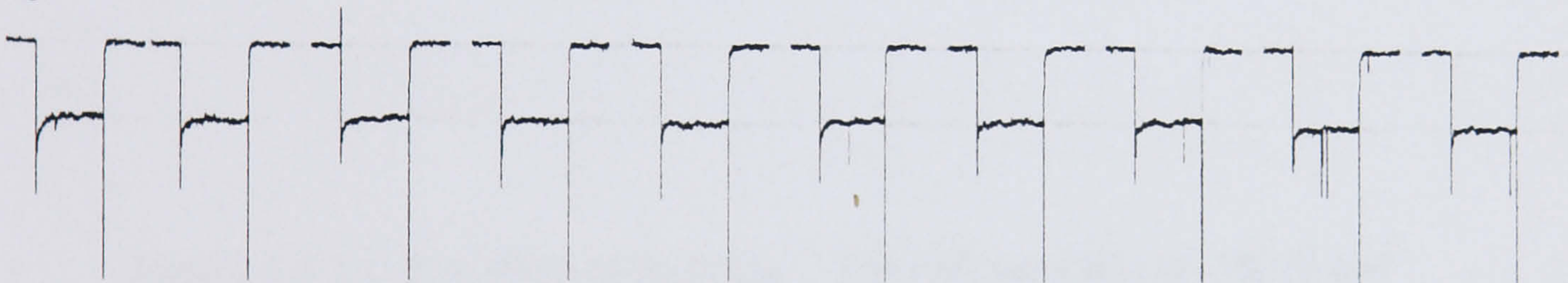


Figure 4.3.3.1: The effect of barium on the background IK1 current in neonatal rat atrial myocytes. A shows 10 control steps. B shows the onset of $3\mu\text{M}$ barium block which begins on the sixth pulse; C shows the block after 4 minutes. Cells were held at -50mV and stepped down to -120mV for 1 second. Steps were separated by 1 second. Barium was bath applied.

level.

Mention holding current

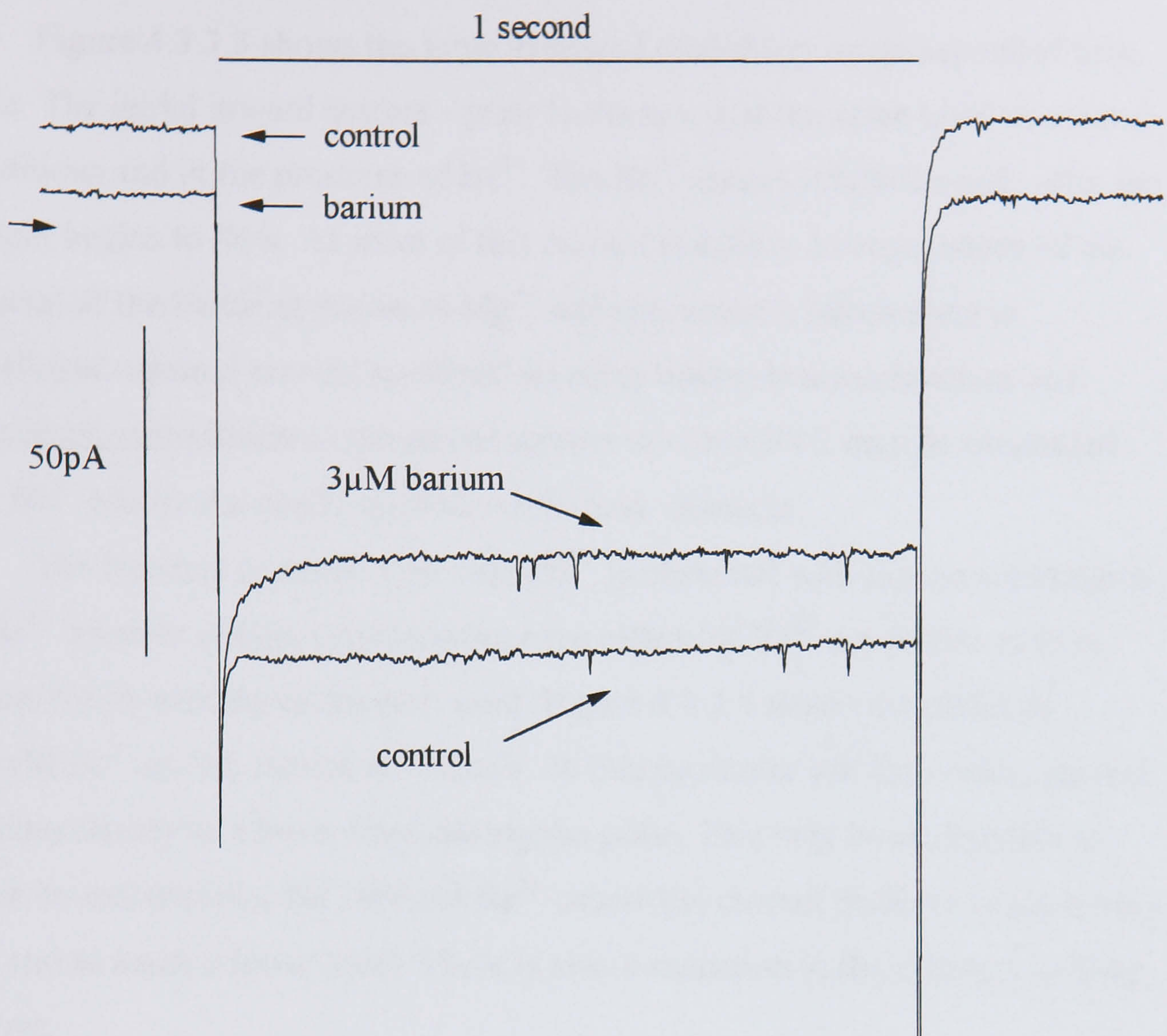


Figure 4.3.3.2: The effect of $3\mu\text{M Ba}^{2+}$. The cell were held at -50mV and a voltage step was applied to -120mV . The traces above are the average from 10 such steps, under control conditions and in the presence of $3\mu\text{M}$ barium. The barium was bath applied and the cell was exposed for 4 minutes. The arrow on the left indicates the zero current level.

Figure 4.3.3.3 shows the same averaged recordings on an expanded time scale. The initial inward current - prior to decay - is at the same level in control conditions and in the presence of Ba^{2+} . The Ba^{2+} causes inhibition only after the current begins to flow. As most of this current is mainly a consequence of the reversal of the blocking action of Mg^{2+} and polyamines, (since there is significant outward current at -50mV so many channels are unblocked and contribute immediately to the ohmic current at -120mV) it may be concluded that Ba^{2+} blocks the newly opened, unblocked, channels.

The increase in decay seen with Ba^{2+} is observed with low concentrations of Ba^{2+} whereas at high concentrations the effects of Ba^{2+} are so fast as to be unresolvable with the techniques used. Figure 4.3.3.4 shows the effect of 100 μM Ba^{2+} on the current at -120mV. In this particular cell the control current declines slowly to a lower level during the pulse. This may be attributable to block by extracellular Na^+ . 100 μM Ba^{2+} causes the current decay to become very fast and to reach a lower level. There is also a reduction in the outward holding current.

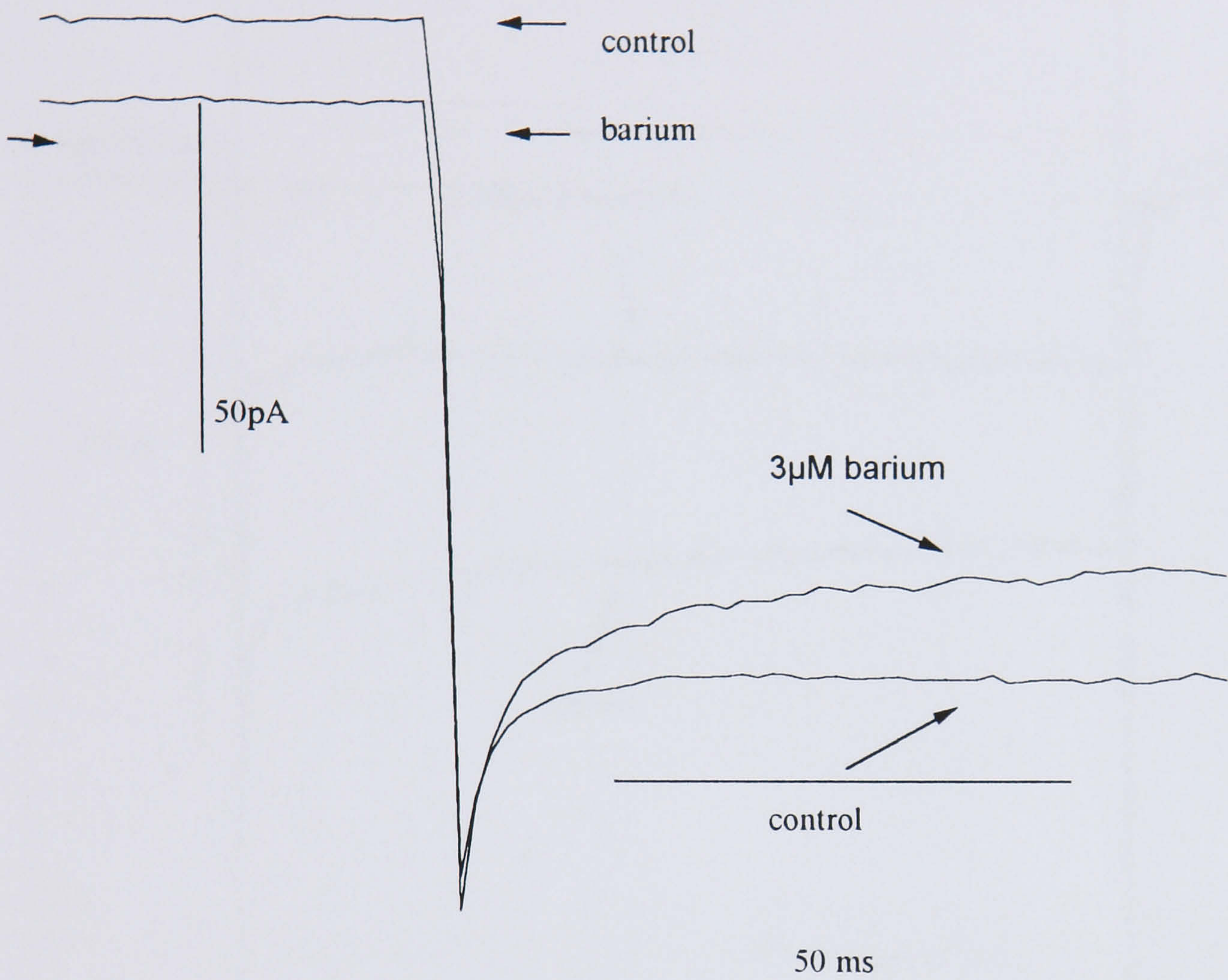


Figure 4.3.3.3: Parts of the current traces shown in Figure 4.3.3.2 but on an expanded scale. This shows the control current decay and the effect of $3\mu\text{M}$ barium. The traces are averages of 10 and are in response to voltage steps to -120mV from a holding potential of -50mV .

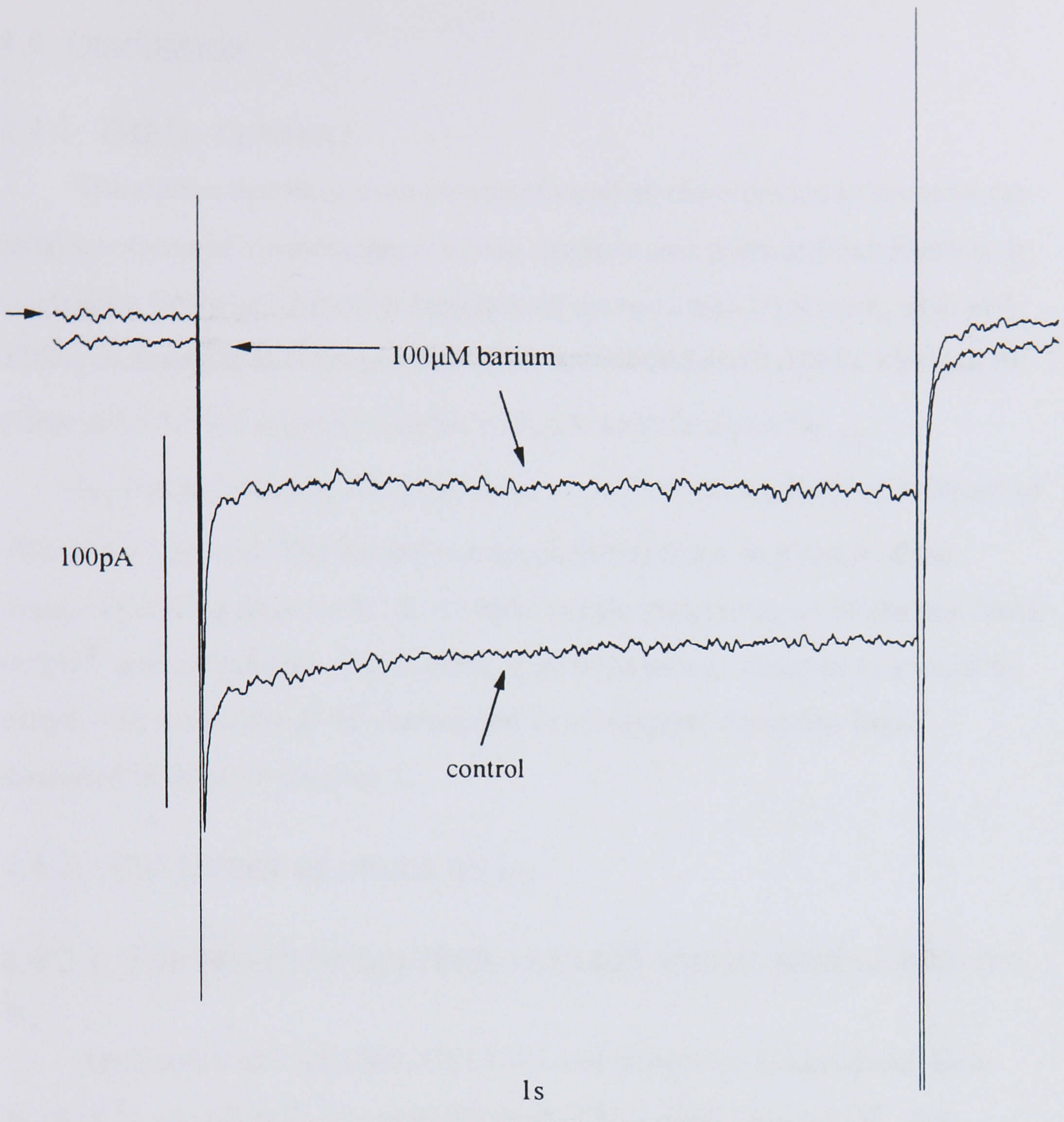


Figure 4.3.3.4: The effect of a high concentration of barium on the background current. The control current shows a decay to a steady state level. 100 μM barium reduces the steady state level and also seems to increase the initial decay. Cells were held at -50mV and 10 voltage steps to -120mV applied, of 1 second duration. The averages of these 10 traces are displayed. The barium was bath applied for 4 minutes. The arrow on the left of the trace indicates the zero current level.

4.4: DISCUSSION.

4.4.1: THE I_{K_1} CURRENT.

The results that have been presented confirm the presence in neonatal rat atrial myocytes of a conductance which displays strong inward rectification. It is possible, however, that other background currents may be present, such as I_f , $TW I_{K_1}$ or some basal G-protein-activated current (Ito *et al.*, 1991). The lack of effect of UCL1880 suggests that there is little contribution of $I_{K_{ACh}}$.

I_{K_1} was activated by stepping down to -120mV from a holding potential of -50mV for 1 second. The inward current observed arises as a result of the change in driving force on K^+ at -120mV and the rapid reversal of channel block by Mg^{2+} and polyamines. The current, once established, decayed to a variable extent. The activation of the current and its subsequent decay has been discussed in detail in Chapter 1.

4.4.2.: THE EFFECT OF DRUGS ON I_{K_1} .

4.4.2.1: THE EFFECT OF UCL1880, UCL1495 AND GLIBENCLAMIDE ON I_{K_1} .

Application of UCL1880, UCL1495 and glibenclamide produced little block of I_{K_1} at -120mV. The concentration of UCL1880 used - 2 μ M - was effective in blocking the G-protein gated current as described in Chapter 3. It can be seen from Table 4.3.1 that at 2 μ M UCL1880 produces no significant effect at -120mV and even 10 μ M UCL1880 results in block of only ~ 20%. For the purposes of this study, the main and important point is that at the concentrations effective on the G-protein-gated current there is no block of the I_{K_1} current by UCL1880.

UCL1495 was tested at 2 μ M. This concentration, which caused approximately 90% block of the G-protein-gated current, caused little (5%) block of I_{K_1} at -120mV. Nor was there evidence for any significant block of I_{K_1} at -120mV by either 20 μ M or 50 μ M glibenclamide.

The overall conclusion is that the concentrations of UCL1880, UCL1495 and glibenclamide which are effective in blocking the G-protein-gated current (Chapter 3) did not inhibit the background I_{K_1} .

The effects of the drugs on the holding current were also examined but the difficulty here was that the current flowing at -50mV was no greater than 10pA and varied considerably from cell to cell. Any blocking action was therefore difficult to evaluate. It is presumed that much of the holding current at -50mV flows through I_{K_1} channels though, as noted earlier, there may be some basal activity of the G-protein-gated current (Ito *et al.*, 1991). If there was a significant portion of the holding current due to basal G-protein activity then some block by the drugs at the concentrations tested would have been expected, as these were effective in blocking the adenosine-activated current as described in Chapter 3. However, the holding current at -50mV was too small and variable to allow this to be evaluated with any degree of certainty.

The effects of glibenclamide are thought to occur by its interaction with the sulphonylurea receptor thus if the SUR was to combine with the I_{K_1} channels then block by glibenclamide might be possible. The lack of effect of glibenclamide on the I_{K_1} channels indicated that there is little or no association of the SUR with I_{K_1} channels. This is discussed further in Chapter 6.

4.4.2.2: THE BLOCK OF I_{K_1} BY Ba^{2+} .

The block of the current by Ba^{2+} was as expected. The decay observed in the control currents increased in the presence of Ba^{2+} . Ba^{2+} is known to block the open channels in a voltage-dependent manner, which is in keeping with the observed voltage-dependence in the present study. Any block of the current at -50mV was difficult to measure as previously explained so that a complete concentration-response curve was not determined. 300 μ M Ba^{2+} caused a block of $60 \pm 10\%$ at -50mV and a somewhat greater block of $77 \pm 12\%$ at -120mV.

It has been reported that the block by Ba^{2+} is reduced when the external $[K^+]$ is increased (DiFrancesco *et al.*, 1984). This suggests that Ba^{2+} and K^+ compete for the same site within the channel which is in keeping with the

evidence that Ba^{2+} blocks the open channels. The potential-dependence of the block is a consequence of the blocking site residing within the voltage gradient across the cell membrane. The Ba^{2+} is attracted by the electrical field of the membrane into the channels where it binds. This resembles H^+ block of Na^+ currents in frog nodes of Ranvier (Woodhull *et al.*, 1973).

The decay occurring during the control hyperpolarising pulse may be a result of an intrinsic decay and/or a block by extracellular cations such as Na^+ . The results show that Ba^{2+} increases the rate of decay. The block by Ba^{2+} is time-dependent in rabbit Purkinje fibres and frog skeletal muscle (Carmeliet and Mubagwa, 1986, Standen and Stanfield, 1978). Ba^{2+} has been shown to cause a block of the open channels in guinea-pig heart cells decreasing the mean open time (Sakmann and Trube 1984) without an effect on the current amplitude. Single channel studies show that the Ba^{2+} block is voltage-dependent being greater at negative potentials (Bechem *et al.*, 1983).

The log concentration-response curve for the findings with Ba^{2+} provided an estimate for the IC_{50} value which at ~~16.8~~ μM , although in the low μM range and not at all precise, was not as low as other reported values such as $2.2\mu\text{M}$ at -125mV in bovine pulmonary artery endothelial cells (Voets *et al.*, 1997), $12\mu\text{M}$ at -65mV in skeletal muscle (Standen and Stanfield, 1978) and $1.3\mu\text{M}$ at -120mV in Purkinje fibres (Carmeliet and Mubagwa, 1986). Also it was noted that the log concentration-response curve was flat with a low n_{H} value of 0.28. It is possible then that there are two components of the background current, one having a greater susceptibility to block by Ba^{2+} than the other. It is conceivable that I_{f} contributes to the background current observed. In addition there may be some basal G-protein turnover which would also affect the results. However UCL1880, which was observed to be potent (IC_{50} of $0.18\mu\text{M}$) on the G-protein-activated current in Chapter 3, at $2\mu\text{M}$ did not block the background current, thus ruling out a significant contribution of this current to the background current. Another current which may also contribute to the background current investigated is TWI_{K1} , a weak inward rectifier which is blocked by Ba^{2+} with an IC_{50} of $100\mu\text{M}$ (Chapter 1, section 1.5.2).

To summarise, this study of the I_{K_1} current has shown that the compounds which are effective against the G-protein-gated current have a minimal blocking action on the I_{K_1} current and therefore exhibit a considerable degree of selectivity in this regard. The results also indicate that the Ba^{2+} block of I_{K_1} channel is similar to that reported in other cell types and species. This suggests that though the background current investigated might be composed of more than one component, the major contributor is I_{K_1} .

CHAPTER FIVE: THE LEVCROMAKALIM-INDUCED
POTASSIUM CURRENT IN NEONATAL RAT
VENTRICULAR MYOCYTES.

CHAPTER FIVE: THE LEVCROMAKALIM-INDUCED POTASSIUM CURRENT IN NEONATAL RAT VENTRICULAR MYOCYTES.

5.1: INTRODUCTION

A reduction in the action potential duration and the associated increase in the whole cell outward current due to metabolic inhibition in cardiac tissue has been the focus of much research in the last few years (Sanguinetti *et al.*, 1988; Findlay, 1989, 1993; Wilde *et al.*, 1990; Nakaya *et al.*, 1991; de Lorenzi *et al.*, 1994). These characteristics have been attributed to the opening of K_{ATP} channels which were first investigated at the single channel level in cardiac tissue by Noma (1983). The conductance of the channel in cardiac myocytes in symmetrical 140mM K^+ is 70 -90pS (Noma, 1983, Horie *et al.*, 1987, Terzic *et al.*, 1995). This channel has also been found in other tissues including pancreatic β -cells, skeletal muscle, smooth muscle, renal tubule cells, and some central nervous system neurons. The single channel conductance shows variation, for example in skeletal muscle the conductance is between 40 and 60pS and in pancreatic β -cells it is between 50 and 75pS (Davies *et al.*, 1991, Terzic *et al.*, 1995, Isomoto *et al.*, 1997). K_{ATP} channels are weak inward rectifiers (for review and additional references see Nichols and Lopatin 1995, Doupnik *et al.*, 1995, and Introduction).

Subsequent work in cardiac tissue has demonstrated channel opening in response to potassium channel openers (KCOs) such as cromakalim, an experimental antihypertensive agent which is active in the μ M range (Sanguinetti *et al.*, 1988; Escande *et al.*, 1988; Osterrieder *et al.*, 1988; Findlay *et al.*, 1989) and levcromakalim which is the active enantiomer of cromakalim and has an IC_{50} of 1.9 μ M in guinea-pig papillary muscles (Findlay *et al.*, 1989). The current activated by KCOs can be blocked by sulphonylureas such as tolbutamide (Findlay *et al.*, 1989) and glibenclamide which at low concentrations are generally considered to be highly selective for $I_{K_{ATP}}$ (Sanguinetti *et al.*, 1988). Tolbutamide is ~ 50 times less potent in the heart than in β -cells with an IC_{50} for block of whole cell K_{ATP} current of 7 μ M in β -cells

and 400 μ M in cardiac cells (Belles *et al.*, 1987). Glibenclamide blocks the atrial channel with an IC_{50} of 5-20 μ M whereas in pancreatic β -cells the IC_{50} is 4-50 nM (Zunkler *et al.*, 1988a,b).

The exact nature of the K_{ATP} in smooth muscle has been complicated by the possibility of there being two different types of channel. In some studies on smooth muscle cells it was observed that the channels activated by metabolic inhibition with cyanide and those activated by KCOs such as pinacidil had similar single channel characteristics and were blocked to the same extent by glibenclamide (Zhang and Bolton, 1995, Teramoto and Brading, 1996). In contrast, however, some studies have suggested that the channels activated by KCOs are not the same as those activated by metabolic inhibition. It has been suggested that there are two types of channels, one of which is the K_{ATP} channel recognised in other tissues and the other a channel which is regulated by intracellular nucleoside diphosphates and is the target for KCOs (K_{NDP}) (Beech *et al.*, 1993b, Zhang & Bolton, 1996). This argument stems from the difference in sensitivity to sulphonylurea block of the current activated either by KCOs or by metabolic inhibition and this has been observed mainly for the K_{ATP} in some studies on smooth muscle cells (Beech *et al.*, 1993b).

K_{NDP} is not thought to exist in the heart. However it was observed in rat ventricular myocytes that activation of the current by rilmakalim was blocked by glibenclamide with an IC_{50} of 8 nM whereas block of the current induced by metabolic inhibition with 2-deoxy-D-glucose occurred with an IC_{50} of 480 nM (Krause *et al.*, 1995). It has been proposed that the increase in $[ADP]_i$ or the decrease in $[ATP]_i$ during ischaemia and hypoxia (Venkatesh *et al.*, 1991) affects the sensitivity of the current to sulphonylurea block (Findlay 1993).

5.2: AIMS

The K_{ATP} channels investigated in cardiac myocytes were activated by the KCO levcromakalim. The drugs which had been tested on the G protein-gated current (Chapter 3) and the background inwardly rectifying current (Chapter 4) were also tested on the current activated by levcromakalim. The results were to be compared with those from the other two chapters in order to

assess the selectivity of the blocking compounds between the three different inward rectifiers. It was also hoped that the results would prove useful in determining whether the activation of $I_{K_{ACh}}$ by adenosine includes a component of $I_{K_{ATP}}$ (Kirsch *et al.*, 1990 Li *et al.*, 1995, and Chapter 3).

5.3: RESULTS:

5.3.1: THE LEVCROMAKALIM-INDUCED CURRENT.

In all experiments ATP was omitted from the internal pipette solution to avoid any inhibition of the current activated by the opener. This proved effective in allowing the cells to respond to levcromakalim; channel run down was not normally a problem.

Initially it was the aim to investigate $I_{K_{ATP}}$ in atrial myocytes. However, the application of 10 μ M levcromakalim did not activate an outward current in these cells (though one sometimes developed spontaneously – see section 5.3.3). It has been observed that the KCOs pinacidil and levcromakalim activate an outward current in rabbit ventricular myocytes but have no effect in rabbit atrial myocytes whereas in both guinea-pig atrial and ventricular myocytes the KCOs were effective (Ogbaghebriel & Shrier, 1995). This would suggest a tissue (and species) difference in response to KCOs and possibly also a differential distribution of K_{ATP} channels. Experiments were accordingly made on ventricular cells which were isolated from 1 day old rats adopting the procedure used in the isolation of atrial myocytes.

It has already been found that the KCOs are effective within the μ M range on cardiac tissue (Sanguinetti *et al.*, 1988, Findlay *et al.*, 1989, Krause *et al.*, 1995). Accordingly, 10 μ M levcromakalim was initially applied in an attempt to activate the current. This concentration did not give a consistent activation. In 7 out of 20 cells tested, the current activated was 100 ± 23 pA in amplitude; the remaining cells did not respond. 1 μ M glibenclamide blocked the current activated by 10 μ M levcromakalim by $81 \pm 11\%$ (n=7) and 2 μ M UCL1880 blocked the current by $40 \pm 12\%$.

Increasing the concentration of levcromakalim to 50 μ M resulted in the consistent activation of an outward current of 196 ± 27 pA amplitude (n=46). Figure 5.3.1.1 shows a representative trace of this response. The current was activated after a short delay of approximately 5-10 seconds in this particular cell, which is most probably due to the time taken for the drug solution to reach the cell. However it should be noted that activation of this current has been observed to occur after a delay (Sanguinetti *et al.*, 1988, Krause *et al.*, 1995) and in some of the ventricular cells tested the current activation was delayed by up to 1 minute. Rarely, it took up to 5 minutes for the current to be activated and if current activation was not observed after 5 minutes the cell was discarded.

For the cell shown in Figure 5.3.1.1 the current rises quickly and reaches ~70% of the peak current within 10 seconds. It is well maintained throughout the levcromakalim application. The subsequent application of 1 μ M glibenclamide in the continued presence of levcromakalim resulted in an almost immediate reduction in the outward current. The recovery from the block also began fairly quickly, within 20-30 seconds. The delay is most probably due to slow washout of the rather high concentration of glibenclamide that has been applied. In this example the current did not return to the level recorded prior to application of levcromakalim and this may be an instance where run down of the levcromakalim-induced current occurred.

Figure 5.3.1.2 shows the current -voltage relationship from the cell illustrated in Figure 5.3.1.1. It can be observed that the current in the presence of 50 μ M levcromakalim has increased in both the inward and outward directions and the subtracted levcromakalim-induced current is very weakly rectifying. The reversal potential is close to the estimated equilibrium potential for K⁺ set in these cells (-94mV) so that it can be concluded that the current is carried by K⁺.

Because the current activated in the rat ventricular myocytes by 50 μ M levcromakalim was K⁺ selective, displayed weak inward rectification, and was blocked by the sulphonylurea glibenclamide, it can be presumed to be I_{K,ATP}.

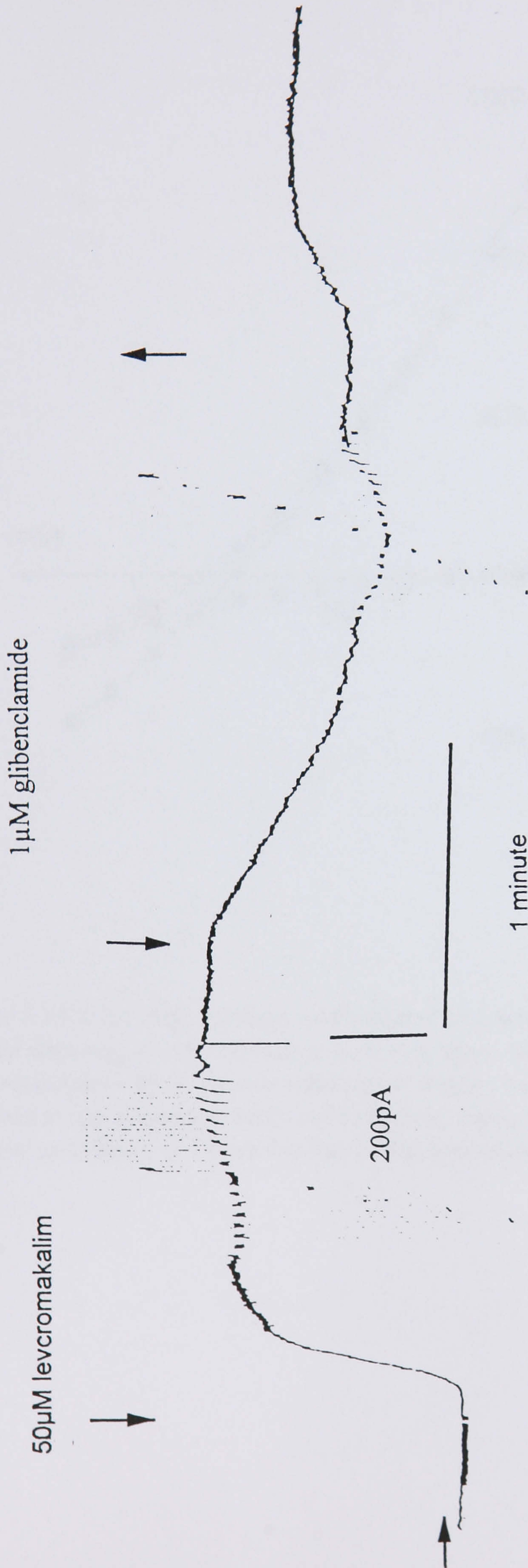


Figure 5.3.1.1: This shows the current trace from a ventricular myocyte held at -50mV . The application of $50\mu\text{M}$ levcromakalim resulted in the activation of an outward current. This current remains activated in the continuous presence of levcromakalim, which is present throughout the remainder of the trace. The application of $1\mu\text{M}$ glibenclamide resulted in the block of the current. The arrow on the right indicates the washout of glibenclamide, and the partial recovery of the current can be seen. The vertical deflections are the currents in response to voltage steps. The arrow on the left indicates the zero current level.

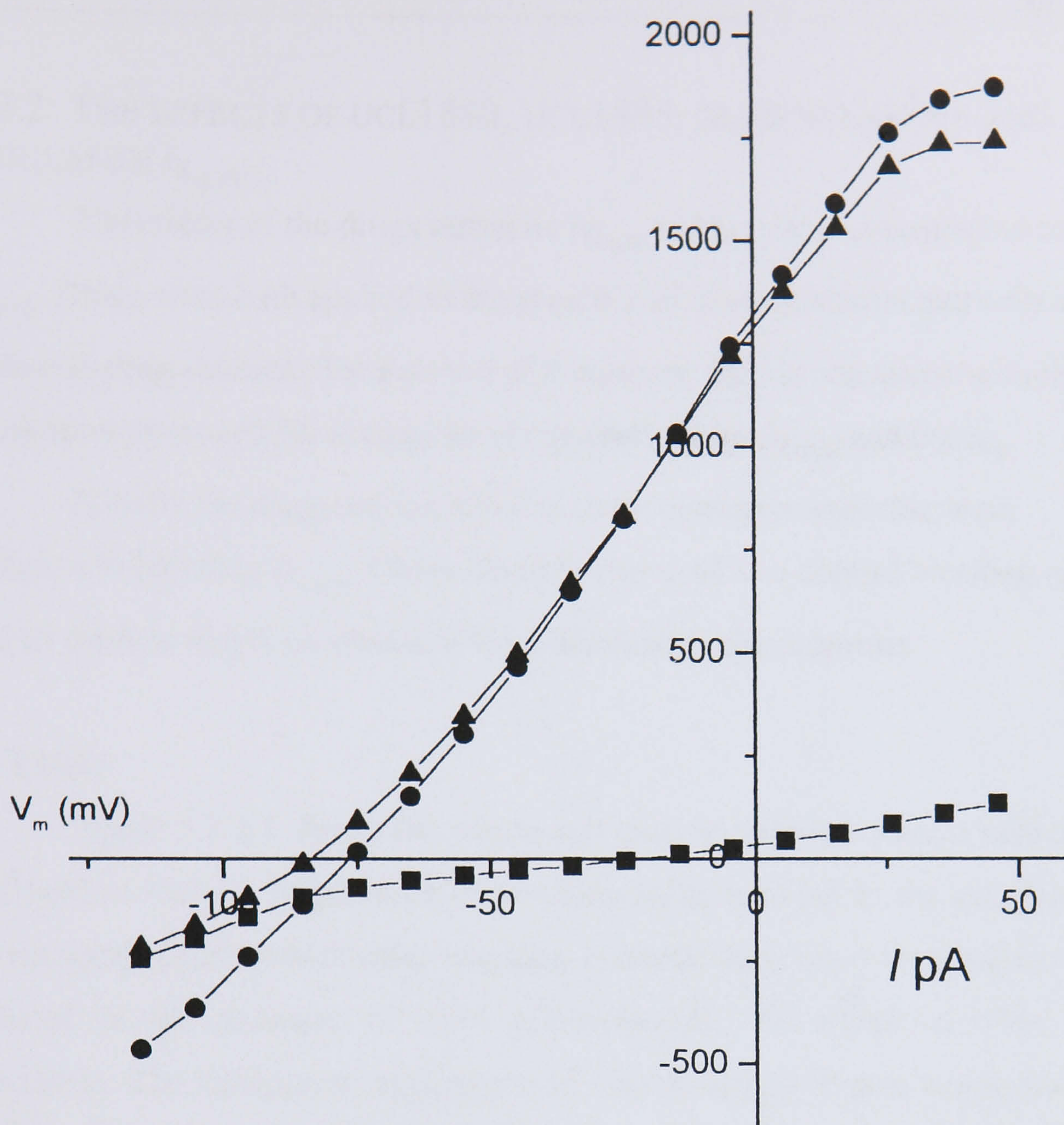


Figure 5.3.1.2. The current-voltage relationship of a rat ventricular myocyte. The background current displaying inward rectification ($\text{---}\blacksquare\text{---}$) is shown with the current in the presence of 50 μ M levcromakalim ($\text{---}\bullet\text{---}$). The subtracted current displays weak inward rectification ($\text{---}\blacktriangle\text{---}$). The cell was held at approximately -50 mV and voltage was stepped from between approximately -120 mV and +50 mV in +10 mV increments. The levcromakalim was bath applied.

5.3.2: THE EFFECTS OF UCL1880, UCL1495, GLIBENCLAMIDE AND BARIUM ON $I_{K_{ATP}}$.

The effects of the drugs tested on $I_{K_{ACh}}$ and I_{K_1} were examined on the $I_{K_{ATP}}$. Drugs were bath applied in the presence of levcromakalim and cells were bathed in drug solutions for a period of 4 minutes. This is consistent with the incubation time used for testing the compounds on the $I_{K_{ACh}}$ and the I_{K_1} .

Initially the drugs were applied at those concentrations that were effective in blocking $I_{K_{ACh}}$. Glibenclamide was used as a control blocking agent and to confirm that K_{ATP} channels were involved in the response.

UCL1880

Figure 5.3.2.1 shows the whole-cell current response from a ventricular cell held at -50mV. Application of levcromakalim resulted in the activation of an outward current which after reaching a steady state level was shown to be reduced in the presence of 1 μ M glibenclamide, the effect of which was reversible. The subsequent application of 10 μ M UCL1880 also resulted in the block of the current and this effect was quick in onset. Washout of this concentration of UCL1880 did not result in recovery of the $I_{K_{ATP}}$ even after 15 minutes.

2 μ M UCL1880 was also tested and the resulting block was not as great as for 10 μ M UCL1880 (see Table 5.3.1). After washout of the 2 μ M UCL1880 the current recovered slowly over a period of 10-15 minutes. In most cases it was found that the current did not regain the pre-UCL1880 level. This could be due either to run down of the whole cell current or to the slowness of the offset of the action of UCL1880.

UCL1495

Figure 5.3.2.2 shows the outward current from a cell which was exposed to two concentrations of UCL1495 in the presence of levcromakalim. An outward current came on in response to levcromakalim after 30-40 seconds but, unlike the current in Figure 5.3.2.1, the outward current in this cell did not

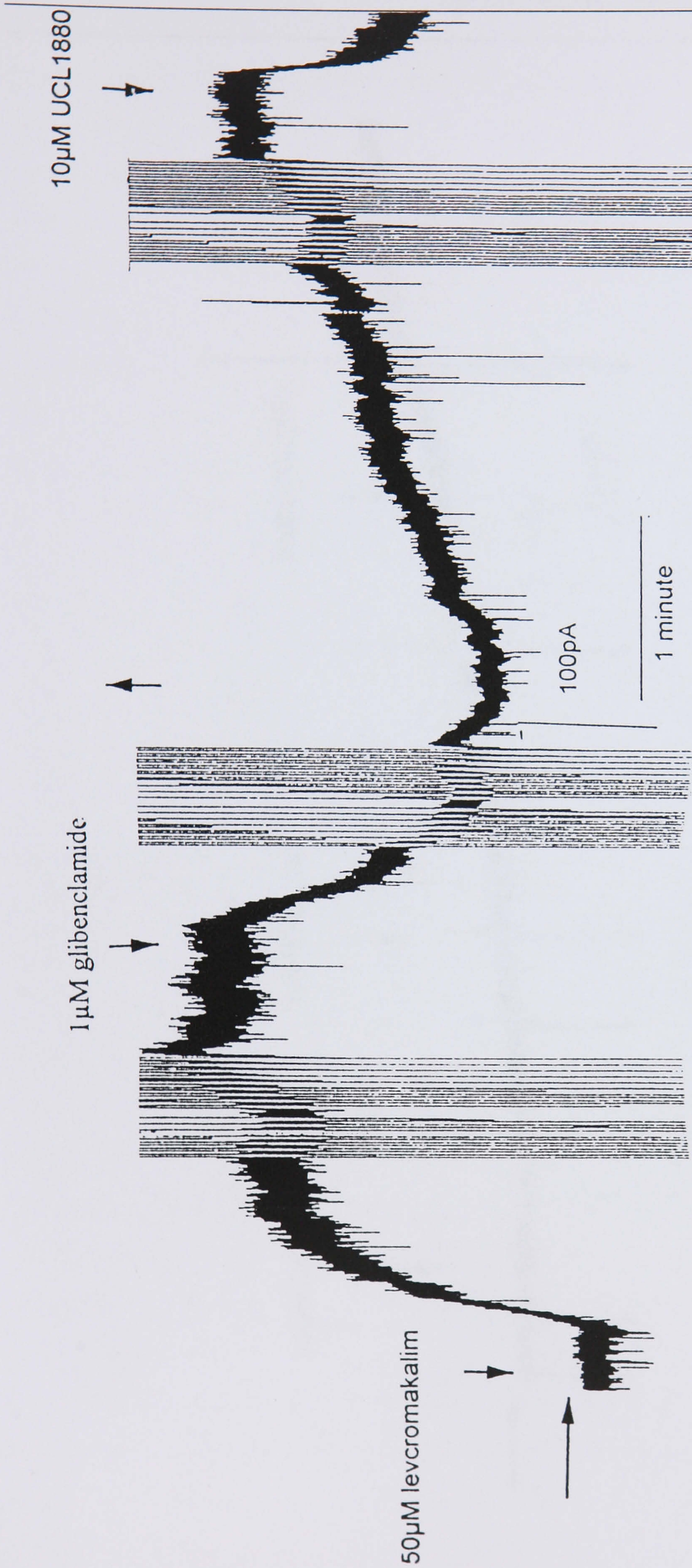


Figure 5.3.2.1: This shows the current activated by 50 μ M levcromakalim. The application of levcromakalim is indicated by the vertical arrow. Levcromakalim is present throughout the remainder of the trace. The current was blocked by 1 μ M glibenclamide and the washout of glibenclamide, indicated by the upward arrow, led to recovery. 10 μ M UCL1880 also blocked the current. There was no recovery from the effect of UCL1880. The vertical deflections are the currents in response to the application of voltage steps. Drug applications are indicated by the vertical downward arrows. The arrow to the left of the trace indicates the zero current level.

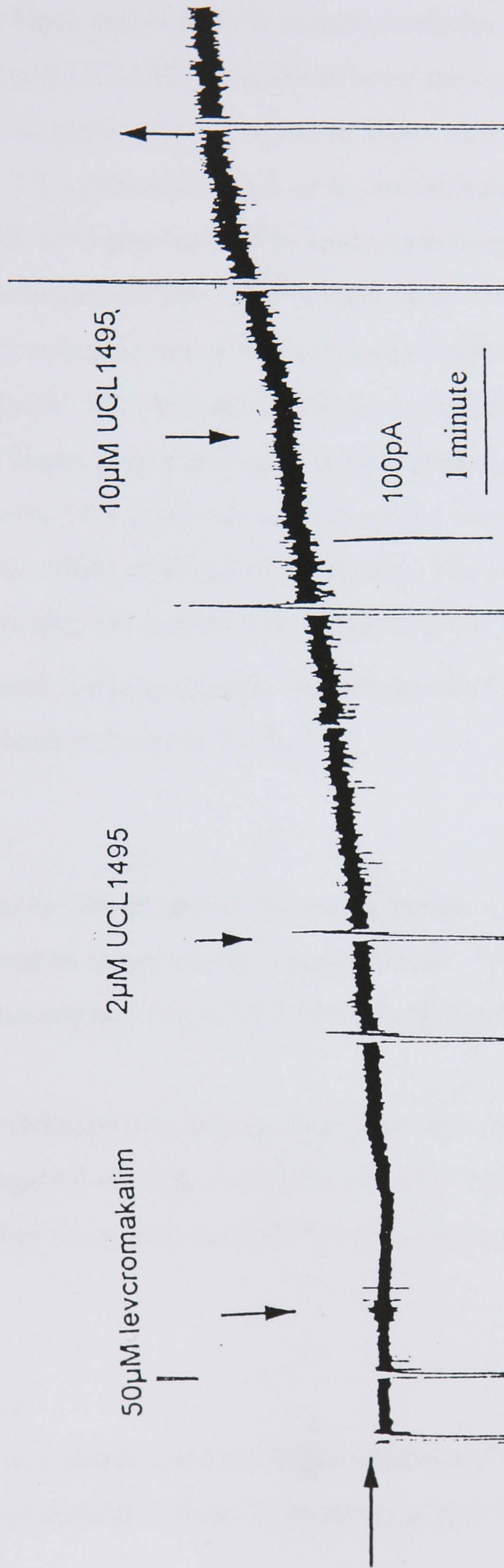


Figure 5.3.2.2: This shows the blocking action of UCL1495. The application of 50µM levcromakalim is indicated by the vertical arrow. Levcromakalim is present throughout the remainder of the trace. The time of application of 2µM UCL1495 is shown. 10µM UCL1495 was applied cumulatively. The current has not been blocked but continues to increase in the presence of UCL1495. The upward arrow indicates the washout of UCL1495. Subsequent application of 1µM glibenclamide resulted in the block of the current. The vertical deflections are currents in response to voltage ramps. The arrow on the left indicates the zero current level. The cell was held at -50mV and all drugs were bath applied.

have a peak but instead gradually increased. Application of 2 μ M UCL1495 did not result in any block and in fact the current continues to rise. In this cell the application of 10 μ M UCL1495 scarcely affected the current. The subsequent application of 1 μ M glibenclamide inhibited it by $\sim 60\%$.

Figure 5.3.2.3 shows the whole-cell current from a different cell in which 10 μ M UCL1495 was tested. The outward current came on in response to the application of levcromakalim after a delay of 30-40 seconds and the current increased rapidly over a period of 1 minute and then more slowly. The application of 10 μ M UCL1495 decreased the current at first after which the outward current began to increase again in the continuing presence of UCL1495. After the UCL1495 was washed out the outward current rose sharply (presumably as the effect of UCL1495 reversed). The subsequent application of glibenclamide blocked the current thus confirming that it was $I_{K_{ATP}}$ and that drug solutions were reaching the cell. The results of all the experiments with UCL1495 have been included in Table 5.3.1.

BARIUM

Ba²⁺ was also tested on this current as shown in Figure 5.3.2.4. 300 μ M barium can be seen to cause almost complete block. Also shown are the effect of 1 μ M glibenclamide and 10 μ M UCL1880. Both blocked the current at all potentials.

The log concentration-response curve for the action of Ba²⁺ is shown in Figure 5.3.2.5 together with the results for the other compounds tested. The Hill equation was fitted to the data for Ba²⁺ block giving an estimate for the IC₅₀ of $165 \pm 14\mu$ M.

CETIEDIL

Cetiedil was also tested against this current and it can be seen from Figure 5.3.2.5 that cetiedil is about 2 times less potent than UCL1880 with an IC₅₀ of $\sim 10\mu$ M.

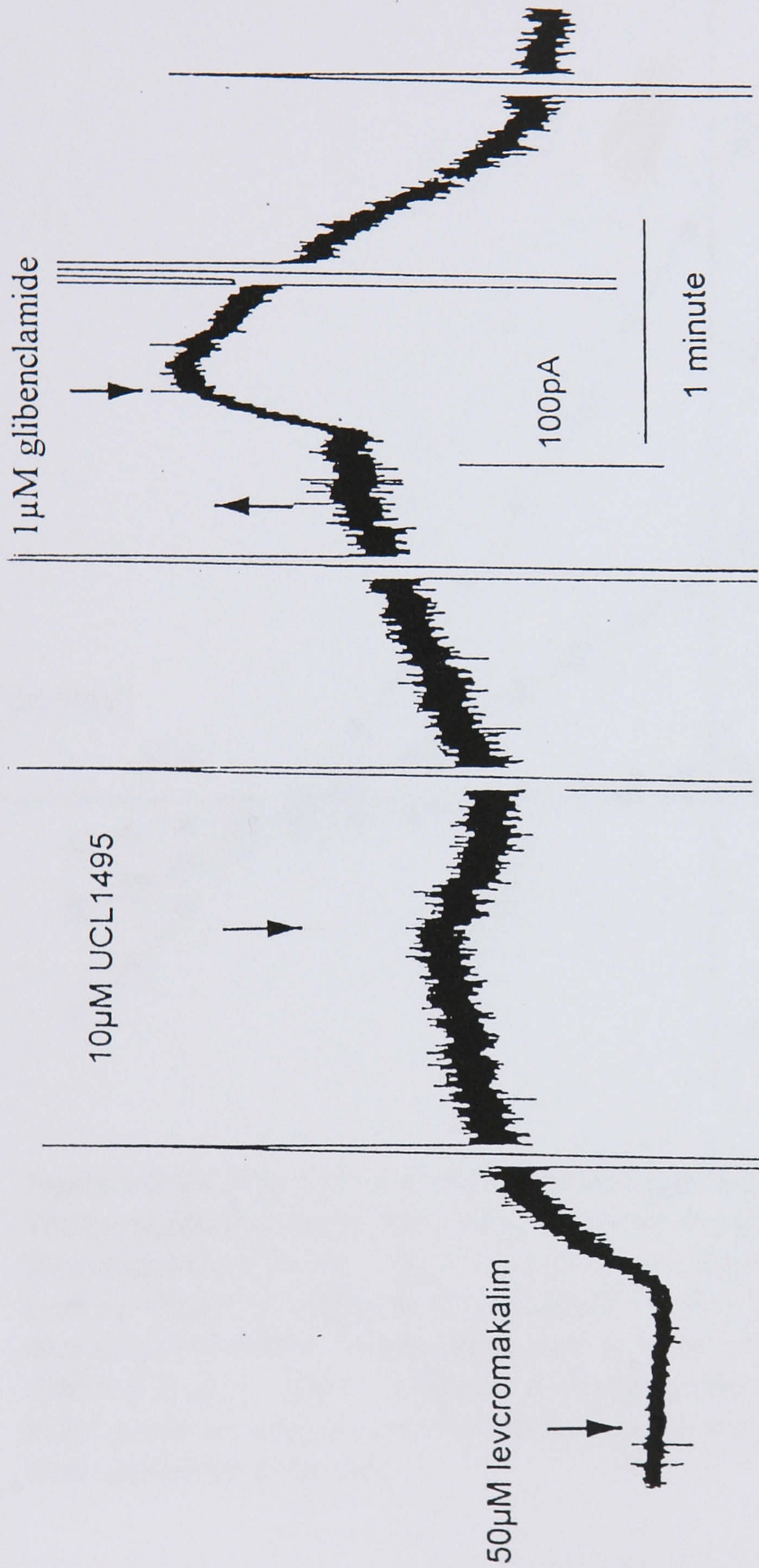


Figure 5.3.2.3: This shows the current activated by 50µM levcromakalim. The application of levcromakalim is indicated by the first vertical arrow. Levcromakalim is present throughout the remainder of the trace. 10µM UCL1495 causes some block but then the current gets bigger. The upward arrow indicated the washout of UCL1495. There is an increase in the current after the washout. Subsequent application of 1µM glibenclamide blocks the current. The vertical deflections are the currents in response to voltage ramps. The cell was held at -50mV. All drugs were bath applied.

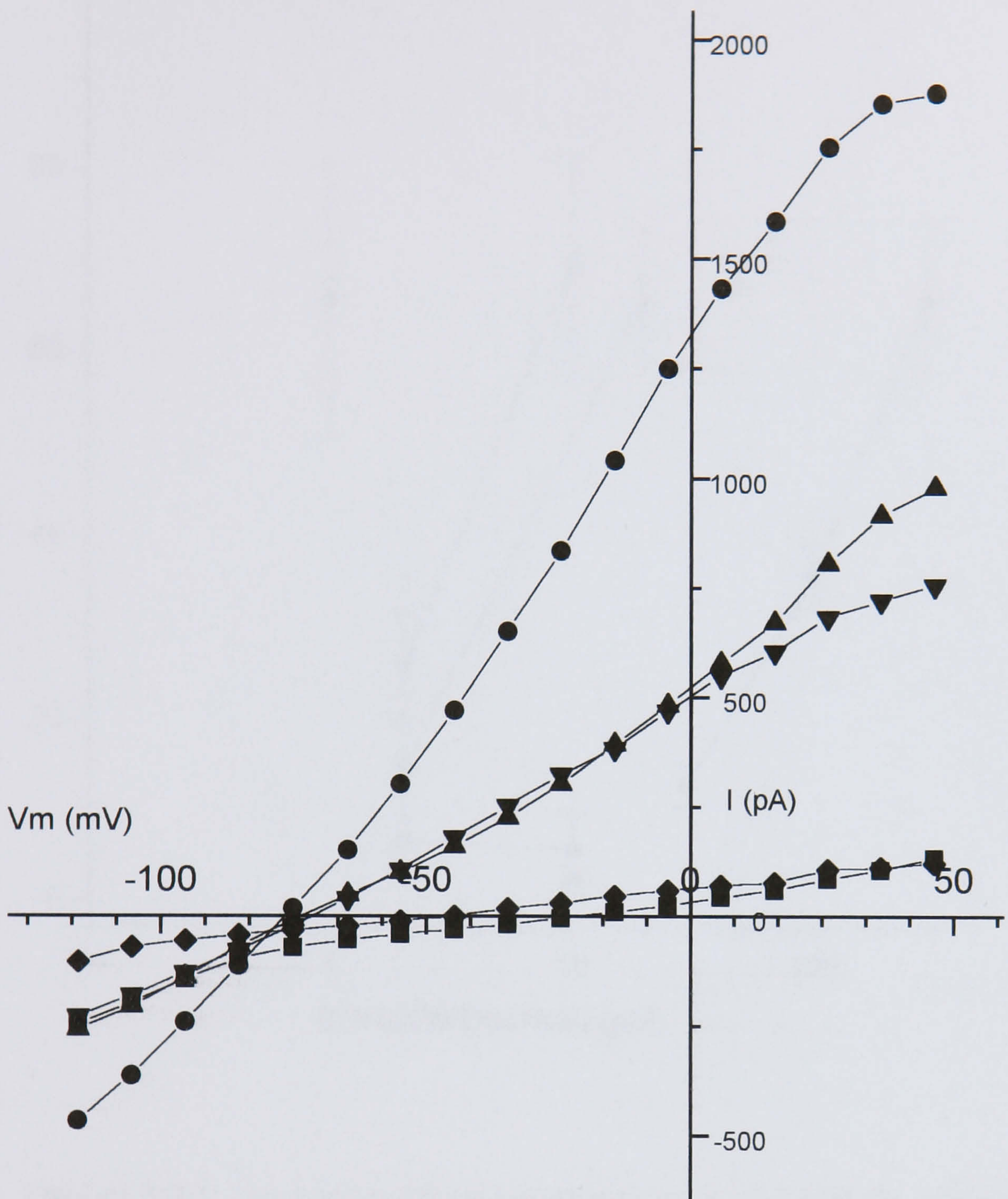


Figure 5.3.2.4: The current-voltage relationship for a rat ventricular myocyte. The background current(—■—) displays inward rectification. The effect of 50 μ M levcromakalim is shown (—●—). Also shown are the effects of 1 μ M glibenclamide (—▲—), 10 μ M UCL1880(—▼—) and 100 μ M barium(—◆—). The cell was held at approximately -50mV. Voltage steps were applied in 10mV increments, down by -70mV and up by +90mV, relative to the holding potential. Blockers were applied in the presence of levcromakalim. Current-voltage traces were recorded 4 minutes after application of blocker.

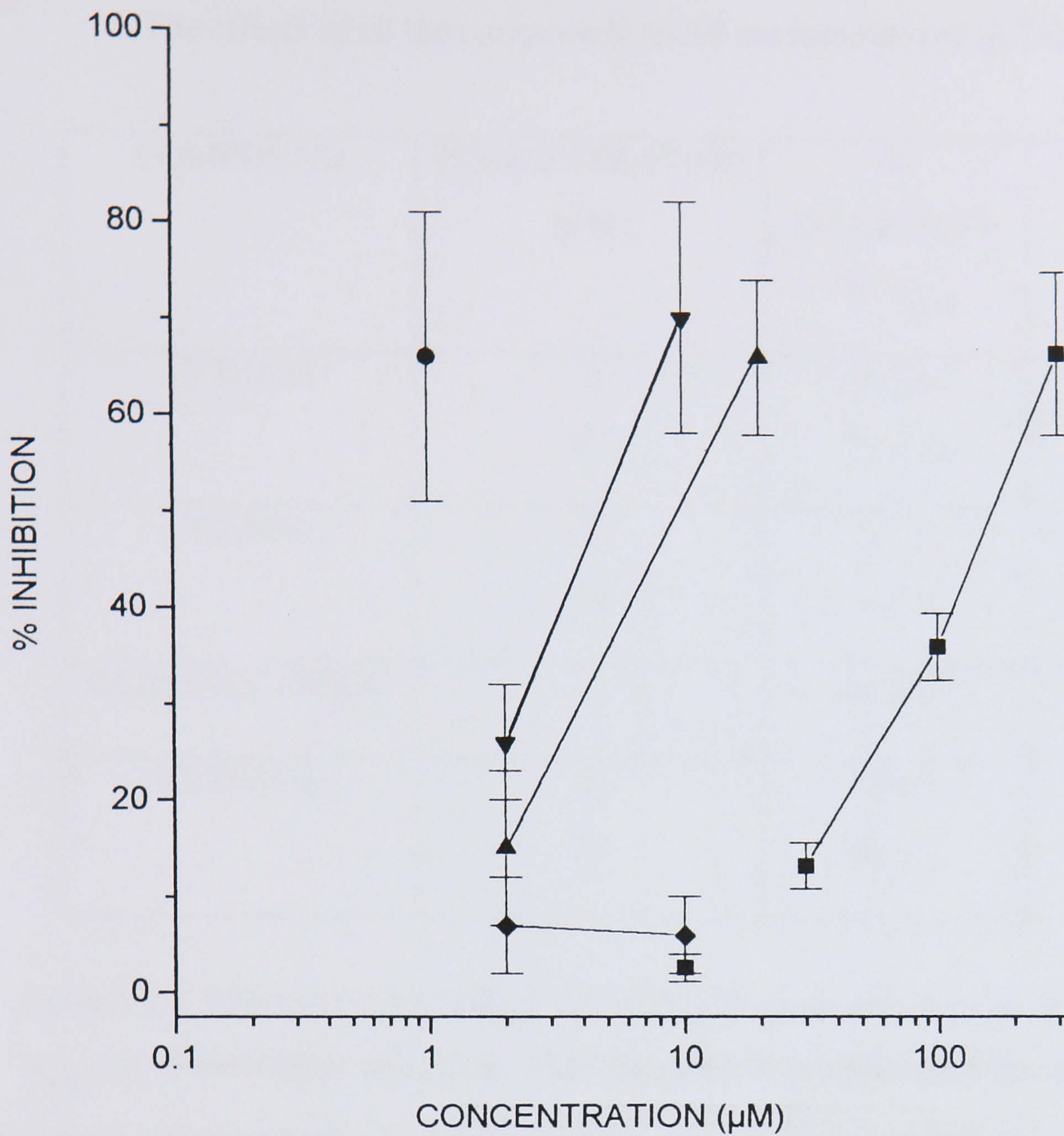


Figure 5.3.2.5: Log-concentration response curves showing the effects of barium (—■—), glibenclamide (—●—), cetiedil (—▲—), UCL1880 (—▼—) and UCL 1495 (—◆—) on the current induced by 50 μM levcromakalim in rat ventricular myocytes. Cells were held at -50 mV. Averages are from at least 3 observations. Note that only barium was fitted with a Hill equation giving an IC_{50} value of $165 \pm 14 \mu M$, and a Hill slope of 1.2 ± 0.1 .

The effects of all the compounds tested are summarized in Table 5.3.1.

COMPOUND	CONCENTRATION (μ M)	% INHIBITION OF $I_{K_{ATP}}$	n
UCL1880	2	26 ± 6	9
	10	70 ± 12	4
UCL1495	2	7 ± 5	7
	10	6 ± 4	3
GLIBENCLAMIDE	1	66 ± 15	7
CETIEDIL	2	15 ± 8	3
	20	66 ± 8	10

Table 5.3.1. Effects of UCL1880, UCL1495, glibenclamide and cetiedil on $I_{K_{ATP}}$ in rat ventricular myocytes. The values are % inhibitions of the outward current activated by 50 μ M levcromakalim, at a holding potential of ~ -50 mV. Values are averages of the n number indicated in the extreme right hand side column.

5.3.3: A SPONTANEOUSLY DEVELOPING OUTWARD CURRENT IN ATRIAL MYOCYTES.

In atrial cells during some long recordings an outward current developed which was susceptible to block by glibenclamide. Though recordings from these cells were normally discontinued, Figure 5.3.3 shows an outward current developing in a cell after a long recording of ~ 15 minutes. The current increased over a period of 2 minutes and was blocked by 20 μ M glibenclamide. Although such currents appeared after long recordings they were not observed in all long recordings. Thus the testing of drugs on this current was in accordance with the type of experiment being carried out. The current amplitude observed in 13 cells was 159 ± 41 pA and the application of 20 μ M

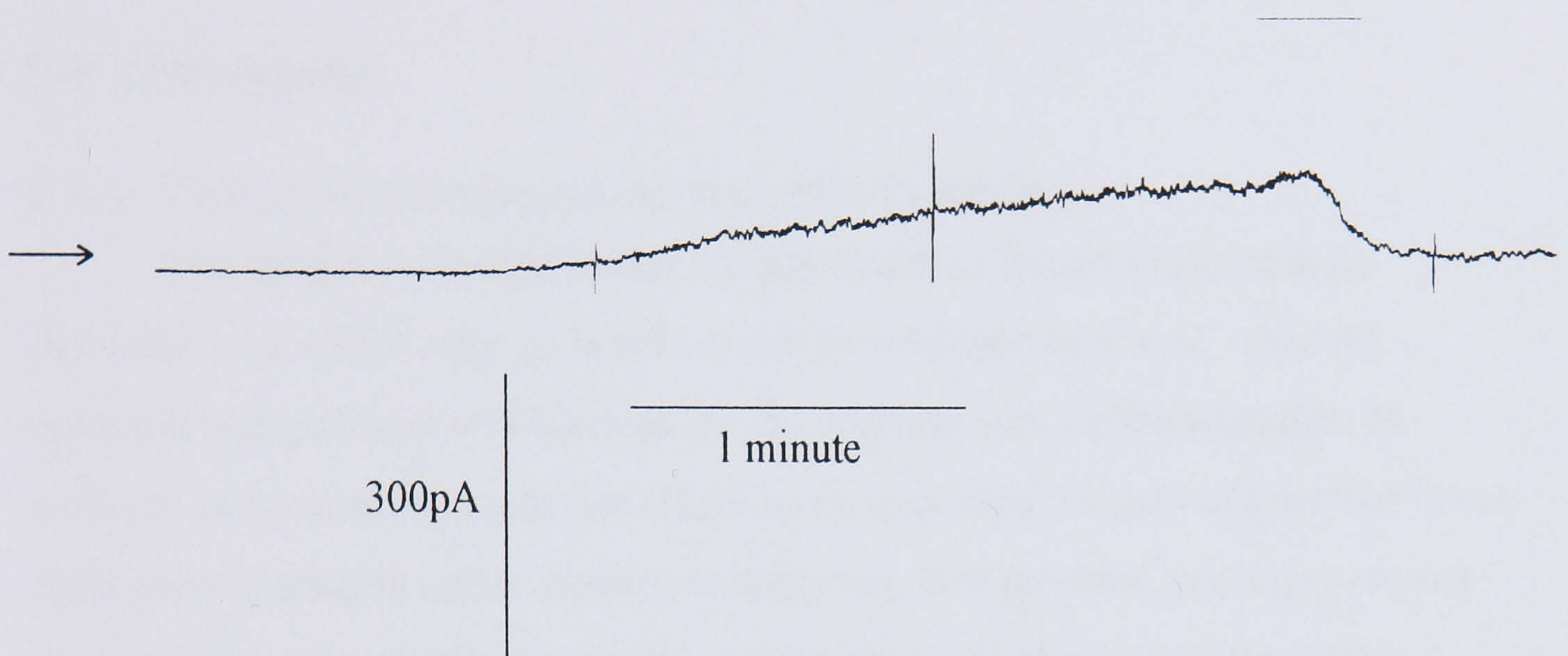


Figure 5.3.3: Effect of glibenclamide on a spontaneous outward current in an atrial myocyte cell. The horizontal bar above the trace indicates the application of 20 μ M glibenclamide. The cell was held at -50 mV. The horizontal arrow on the left indicates the zero current level.

glibenclamide blocked the current by 100% (n=3) though this is a high concentration and possibly enough to block other channels. Lower concentrations of glibenclamide were not tested, and this remains for a future study. This will be discussed further in the Discussion of this chapter (section 5.4).

5.4: DISCUSSION:

5.4.1: THE LEVCROMAKALIM-ACTIVATED CURRENT.

The results presented in this chapter indicate the presence of K_{ATP} channels in rat ventricular cells which can be activated by the K^+ -channel opener levcromakalim and blocked by the sulphonylurea glibenclamide. In contrast, levcromakalim was not effective in activating an outward current in rat atrial cells in studies under identical conditions. It is possible that there are not as many K_{ATP} channels in the atrial as compared with the ventricular cells.

The atrial cells sometimes spontaneously developed an outward current which is blocked by glibenclamide, strongly suggest that rat atrial cells do contain K_{ATP} channels. Thus there may be a tissue-specific action of levcromakalim. There are precedents for this. For example, vascular smooth muscle is 10-30 times more sensitive to the effects of cromakalim than cardiac tissue and also cromakalim is more potent in ferret than in guinea-pig papillary muscle (Quast *et al.*, 1988, Winquist *et al.*, 1989) - thus it may be that K_{ATP} channels in atrial cells are less sensitive to the effects of cromakalim than the channels in ventricular cells. This would merit further study.

$I_{K_{ATP}}$ is weakly rectifying and as seen in Figure 5.3.1.2 at potentials positive to the net reversal potential the current-voltage relationship is almost linear, showing appreciable rectification only at about +25mV. This has been reported previously (Isenberg *et al.*, 1983; Hamada *et al.*, 1990; Wilde *et al.*, 1990; Benndorf *et al.*, 1992; Findlay *et al.*, 1993). In single channel studies it has been reported that K_{ATP} currents are more strongly rectifying when external $[K^+]$ is increased (Krause *et al.*, 1995).

5.4.2: THE BLOCK OF THE LEVCROMAKALIM-ACTIVATED CURRENT.

The results presented in this chapter show that UCL1880 blocks the levcromakalim-induced current at -50mV though at concentrations ~~are~~ higher than those needed to block $I_{K_{ACh}}$ (Chapter Three). The block occurred fairly soon after addition of UCL1880 and there was no recovery from a 10 μ M application even after 15 minutes. It may be recalled from Chapter Three that recovery was also very slow for the block of the G-protein-gated current by UCL1880. To reliably estimate recovery from block by UCL1880 it is essential that the current activated by levcromakalim is maintained at a steady level. However the levcromakalim response seems to suffer varying degrees of rundown which in some cases frustrated a proper assessment of recovery. However channel rundown was not observed in other experiments and also the blocks by glibenclamide, barium, and cetiedil were reversible. Therefore it seems likely that UCL1880 takes a long time to wash out of the lipid membranes or dissociates slowly from the channel protein, especially after high concentrations have been applied.

The structural analogue of cetiedil, UCL1495, caused only a small reduction in the K_{ATP} current at concentrations of up to 10 μ M even after a 4 minute incubation period.

Cetiedil is the lead compound from which UCL1495 was developed and was found to be effective in blocking the G-protein-gated current (Jones, 1995) and the red blood cell Ca^{2+} -activated K^+ channels (Benton, 1995, 1996) and it proved to be effective in blocking the levcromakalim-activated current at concentrations between 2 and 20 μ M.

An interesting observation in atrial cells was the spontaneous activation of an outward current after long recordings (over 10 minutes). This current was susceptible to block by glibenclamide and was thus considered to be the K_{ATP} current. The appearance of this current was an unexpected observation since the application of levcromakalim to the atrial cells did not result in the activation of an outward current. The activation of an outward current after long recordings has been observed in guinea-pig ventricular myocytes (Belles *et al.*, 1987) where the current was blocked by tolbutamide with an IC_{50} of 0.38mM. It was

also observed that increasing the concentration of intracellular ATP delayed the development of the spontaneous current (Belles *et al.*, 1987).

The current which develops spontaneously may be a consequence of cell deterioration and metabolic inhibition. Intracellular nucleotide diphosphates (NDP) build up during metabolic inhibition and are thought to activate the K_{ATP} channels. In smooth muscle cells the characteristics of these channels are similar to the K_{ATP} channels and it is thought that the blocking effects of ATP only occur after activation of the channel by NDPs (Beech *et al.*, 1993). Subsequent studies have revealed two types of ATP-sensitive K^+ channels in smooth muscles of rat portal vein one of which is similar to the 'traditional' K_{ATP} channels and the other is thought to be the one regulated by NDPs, K_{NDP} , (Zhang and Bolton, 1996). It has been suggested that the main target for KCOs in rat mesenteric artery smooth muscle cells is the small conductance K_{NDP} channel (Zhang and Bolton, 1995) (see General Introduction).

This seems to be the situation in smooth muscle cells, but are there also two types of K_{ATP} channels in cardiac cells? Thus far there has been no evidence which indicates the existence of two channel types in cardiac myocytes. The effects of sulphonylureas such as tolbutamide and glibenclamide on the channel activated by metabolic inhibition in the intact heart are less compared to their effects on K_{ATP} channels in excised membrane patches. It is thought that during myocardial ischemia and hypoxia, cytosolic [ADP] rises to the 100 μ M range and this may account for the limited potency of sulphonylureas under these conditions, perhaps by competing for the same binding site (Venkatesh *et al.*, 1991).

It has also been observed in ventricular myocytes from rat hearts that sulphonylurea drugs lose their ability, with time, to inhibit the ATP-sensitive channels activated by metabolic inhibition (Findlay 1993). The ability of tolbutamide and glibenclamide to block the current activated by bimakalim was unaltered. The increase of ADP during metabolic inhibition was thought to be the reason for this change in sensitivity to sulphonylureas (Findlay 1993).

It has also been proposed that the difference in sensitivity of the channels to block by sulphonylureas may be due to the difference in their

activation. Firstly the channels activated by metabolic inhibition have twice the conductance of the channels activated by rilmakalim and, secondly, the current that was activated by rilmakalim was blocked by glibenclamide with an IC_{50} of 8nM whereas the block of the current activated by metabolic inhibition occurred with an IC_{50} of 480nM (Krause *et al.*, 1995). This may reflect the activation of different K_{ATP} channel subtypes or possibly differences in the state of a single channel.

In the present work Ba^{2+} blocked the ATP-sensitive K^+ channels with an IC_{50} of 164 μ M and an n_H value of 1.15 at -50mV. In pancreatic β -cells the blocking action of Ba^{2+} is voltage-dependent, the IC_{50} being 180 μ M at -62mV and \sim 13 μ M at -123mV but it is not thought that K^+ and Ba^{2+} compete for the same site since increasing $[K^+]_o$ did not affect the Ba^{2+} block observed (Takano and Ashcroft 1996). However in frog skeletal muscle, where the block by Ba^{2+} occurred with an IC_{50} of 85mM at 0mV, single channel studies showed that Ba^{2+} decreased the mean channel lifetime of K_{ATP} channels indicating that there was a block of the open channels (Quayle *et al.*, 1988). However, Quayle *et al.*, (1988) did not examine the effects of altering external K^+ on Ba^{2+} block. Thus it is not clear whether or not Ba^{2+} and K^+ compete for the same site within K_{ATP} channels, but it is most likely that there is a block of open channels as is the case for the Ba^{2+} block of I_{K_1} and $I_{K_{ACh}}$ (see Chapter One).

The results obtained in this chapter reveal that the concentrations of glibenclamide needed to block K_{ATP} channels are much less than those needed to block $I_{K_{ACh}}$. Thus it appears that the application of adenosine to rat ventricular cells does not lead to a significant activation of ATP channels.

CHAPTER SIX:
DISCUSSION OF RESULTS.

CHAPTER SIX: DISCUSSION OF RESULTS.

The following section contains a brief discussion of the three different currents investigated in the present study and the effects of the drugs on them. This is followed by a final passage discussing the relative potencies of each drug on these currents.

6.1: THE INHIBITION OF THE G PROTEIN-GATED K^+ CURRENT.

The results presented in Chapter 3 have shown that adenosine when bath applied at $1\mu M$ activates an outward current that displays some fade. Its characteristics have been discussed in Chapter 3. UCL1880, UCL1495, glibenclamide and Ba^{2+} block this current measured at a standard holding potential of $-50mV$.

Of the novel compounds tested the clotrimazole analogue UCL1880 is the most potent inhibitor of the G protein-gated K^+ channel in neonatal rat atrial myocytes, with an IC_{50} of $0.18 \pm 0.02\mu M$. UCL1495 and glibenclamide inhibit with IC_{50} values of 0.46 and $24\mu M$, respectively. It was observed that UCL1880 altered the 'shape' of the current in response to adenosine in that there was an increase in fade compared with that seen in the control response, suggesting a use-dependent action. Importantly, at low concentrations there was no reduction in the peak response but the current decayed to a lower steady state level. This has been observed previously for the effect of clotrimazole on the adenosine-induced change in conductance in rat atrial myocytes (Jones, 1995). Quantifying the ratio of steady state to peak current confirmed that UCL1880 caused a significant increase in fade. UCL1495 was also active in this regard. However, no change in fade was observed for the adenosine-response in the presence of glibenclamide.

In principle, it is possible that UCL1880 and UCL1495 could block the receptor, the receptor-G protein interaction, the activated G protein, the activated G protein-channel interaction or the open channel. There is, however, little or no block of closed channels by these two compounds, as assessed by the unchanged peak current recorded in low concentrations of drug. UCL1880 also

blocked the response to ACh in the atrial myocytes thus increasing the likelihood of a block occurring after receptor activation. The use of GTP- γ -S in some experiments allowed the blocking effect of the compounds on the current to be studied independent of receptor activation. Any inhibition of the current would therefore not be due to a block at the receptor level. UCL1880 did in fact have a blocking effect on the current supported by GTP- γ -S though increased concentrations were required and there was also some suggestion that a residual component of the current remained unaffected. This residual UCL1880-insensitive current could possibly be $I_{K_{ATP}}$ as this has been reported to be regulated by G proteins (Ito *et al.*, 1992, Terzic *et al.*, 1994). However, since 1 μ M glibenclamide was much less effective against this current than it was on the K_{ATP} channels activated by levcromakalim in ventricular cells, (Chapter 5) $I_{K_{ATP}}$ is unlikely to have been involved. This has been discussed further in Chapter 3.

It has previously been observed that UCL1495 inhibited the adenosine-induced increase in conductance in rat atrial myocytes with an IC_{50} of $\sim 0.4 \mu$ M and 1 μ M UCL1495 blocked the GTP- γ -S current in the same tissue by $\sim 40\%$ (Jones, 1995). The IC_{50} value for inhibition of the adenosine response - by $\sim 50\%$ - was estimated in the present study to be 0.46μ M. It would appear that the block of the preactivated current by 1 μ M UCL1495 is less than the expected level of block. This can be taken as additional evidence to support the suggestion that the inclusion of GTP- γ -S in the filling solution causes the activation of another conductance which is insensitive to both UCL1880 and UCL1495. Jones (1995) observed that application of 20 μ M cetiedil resulted in an almost complete block of the GTP- γ -S current, and the block seen in the present study by 20 μ M cetiedil was $\sim 60\%$, - almost maximal. This further supports the idea that there is, in addition to activation of the $I_{K_{ACh}}$, activation of another component insensitive to UCL1880, UCL1495 and cetiedil.

As noted in Chapter 3, control responses to adenosine showed fade. Low concentrations of UCL1880 and UCL1495 increased the fade. Thus it could be speculated that these compounds increase the desensitisation process as

their mechanism of block. For example, β -arrestin has been observed to increase the desensitisation observed in the response to ACh in reconstructed muscarinic receptor/ K^+ channel in Chinese hamster ovary cells (Shui *et al*, 1998). The activation of the muscarinic response and the recovery from desensitisation was also slower in the presence of β -arrestin. In the present study the adenosine response took a long time to recover after washout of high concentrations of UCL1880. This has also been observed for high concentrations of clotrimazole (Jones, 1995). Initial responses to adenosine after washout of the drug still displayed a characteristic fade. It was concluded by Jones (1995) that, since clotrimazole is lipophilic, it dissolves in the membrane lipid and moves from lipid membrane to K^+ channels when these open in response to subsequent applications of adenosine. The outcome is channel block. The same may hold for UCL1880.

Drugs blocking open channels are often found to bind to a site in the pore of the channel which is within the electrical field of the membrane. This would be expected to lead to an effect of voltage on the action of blocking agents that carry an electrical charge. However, the block of the adenosine-current by UCL1880 and UCL1495 displayed little voltage-dependence. It is possible that the drugs are blocking the open channel in an un-ionised form that is not affected by voltage. Alternatively, the concentration attained at the blocking site may not be influenced by the membrane potential.

Glibenclamide blocked the $I_{K_{ACh}}$ current with an IC_{50} of $24\mu M$. There was no evidence of use- or voltage-dependence block. The block of the GTP- γ -S current, as well as that elicited by adenosine, reduces the likelihood that the block occurs at the receptor. An action at the channel and/or the G protein is more probable. The lack of a use-dependent or a voltage-dependent effect does not completely rule out a block of the channel since glibenclamide may block in an un-ionised form, or it may act a site beyond the electrical field. However, as discussed later, glibenclamide is known to interact with the sulphonylurea receptor to block the K_{ATP} current. There is no published work showing glibenclamide blocks K^+ channels directly. Thus it may be that the block of the $I_{K_{ACh}}$ by glibenclamide is due to a cardiac SUR associating with the channel

protein. Alternatively it is possible that adenosine activates K_{ATP} in addition to K_{ACH} channels and this possibility is discussed later.

6.2: THE EFFECT OF DRUGS ON I_{K_1}

Rat atrial myocytes displayed an inwardly rectifying background current. The current pulse induced by a step to -120mV showed varying degrees of inactivation which has been attributed to block by extracellular cations, in particular Na^+ . This has been discussed in Chapter 1 and 4. There was minimal block of this current by UCL1880, UCL1495 and glibenclamide at those concentrations that were effective in blocking the adenosine-induced current. Ba^{2+} blocked the current with an IC_{50} of 17 μ M at -120mV. It was the initial aim to compare any blocking action of the novel compounds with the blocking action of Ba^{2+} to determine if there was any open channel block. 10 μ M UCL1880 blocked the current by ~20% without affecting the kinetics. In contrast low concentrations of Ba^{2+} caused an increase in the decay (see Figure 4.3.2.1 and section 4.3.3). Therefore the small block which occurred by UCL1880 was not the same as the block seen by Ba^{2+} .

6.3: THE EFFECT OF DRUGS ON $I_{K_{ATP}}$

The current activated by levcromakalim in the ventricular cells was blocked by glibenclamide with an IC_{50} of less than 1 μ M. This is less than the reported values of 5-20 μ M for glibenclamide block of these channels in cardiac cells (Zunkler *et al.*, 1988a,b, Rippoll *et al.*, 1990). It is generally thought that the sulphonylurea receptors SUR2A and SUR2B are both present in cardiac tissue. SUR1 is thought to combine with the channel in pancreatic β -cells and to be responsible for the increased potency of glibenclamide in these cells. Since the mRNA for SUR1 has been detected in small amounts in the heart (Inagaki *et al.*, 1995; Anguilar-Bryan *et al.*, 1995) it may be possible that in neonatal rats there is a significant amount of SUR1 combining with Kir6.2 which results in an increased potency of block by glibenclamide.

UCL1880 was also effective in blocking the levcromakalim-induced current, exhibiting an IC_{50} of $\sim 5\mu M$. It is thus less potent on the levcromakalim-activated current than it is on the adenosine-induced current. Cetiedil blocked the current with an IC_{50} of $\sim 10\mu M$ whereas the structural analogue UCL1495 did not cause any block even at $10\mu M$. Ba^{2+} blocked the current with an IC_{50} of $165\mu M$ at $-50mV$ thus being much less potent than on the adenosine-induced current and the background inwardly rectifying current.

6.4: SELECTIVE EFFECTS OF THE COMPOUNDS TESTED BETWEEN $I_{K_{ACh}}$, I_{K_1} AND $I_{K_{ATP}}$

The compounds tested in this study display some degree of selectivity in their blocking effect between the currents that were examined. The order and degree of selectivity and any similarities in blocking actions are discussed in the following sections with reference to each compound.

UCL1880:

It has been shown that UCL1880 is not as effective in blocking I_{K_1} and $I_{K_{ATP}}$ currents in cardiac myocytes as it is in blocking $I_{K_{ACh}}$. The results are summarised in Figure 6.1. $2\mu M$ UCL1880 caused a block of $\sim 90\%$ of the G-protein-gated current but virtually no block of the I_{K_1} current. When tested against the current activated by levcromakalim in ventricular myocytes $2\mu M$ caused a block of 26%. Thus it appears that the UCL1880 has considerable selectivity for $I_{K_{ACh}}$.

The adenosine-induced current is regulated by G proteins and there is evidence to suggest that K_{ATP} channels may also be modulated by G proteins. It might be concluded, therefore, that UCL1880 interacts in some way with G-proteins to suppress the current. However the activation of K_{ATP} channels by levcromakalim does not involve G protein activation and yet they are blocked

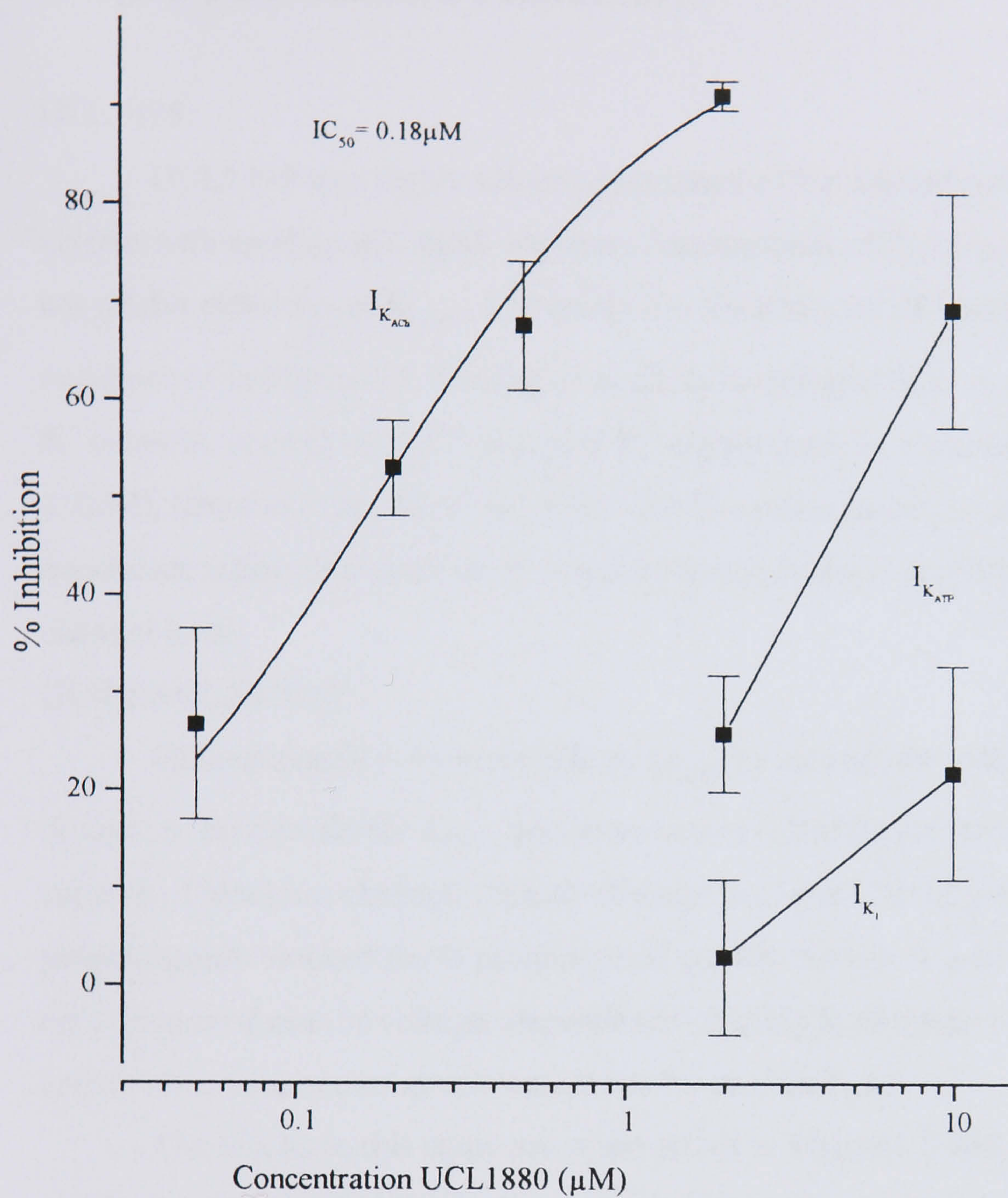


Figure 6.1: The effect of UCL1880 on the three inward rectifiers; the G protein-gated current, the K_{ATP} current and the background inward rectifier, I_{K_I} .

by UCL1880. Also I_{K_1} is not regulated by G-proteins. An action of UCL1880 through G-proteins therefore seems unlikely.

UCL1495:

UCL1495 was highly selective for block of the adenosine-induced current with an IC_{50} of $0.4\mu M$ whereas concentrations of $2\mu M$ and $10\mu M$ did not inhibit either I_{K_1} or $I_{K_{ATP}}$. The results for the effect of UCL1495 are summarised in Figure 6.2. bearing in mind its blocking actions on some other K^+ currents, such as the Ca^{2+} -activated K^+ conductance in erythrocytes (IC_{50} of $1.2\mu M$), (Benton *et al.*, 1994) as well as the G-protein-gated current and its use-dependent action, the block most probably occurs at the G protein and/or the channel level.

GLIBENCLAMIDE:

Glibenclamide is known to block $I_{K_{ATP}}$ by interaction with SUR which is thought to be specific for K_{ATP} , and there is no evidence to indicate that it is capable of blocking channels directly (though see later). In the present study, glibenclamide blocked the G protein-gated current with an IC_{50} of $24\mu M$ with no evidence of use- or voltage- dependence. The block of the levcromakalim-activated current occurs at concentrations lower than $1\mu M$.

The results in this study are summarised in Figure 6.3 and show that $1\mu M$ glibenclamide blocks the K_{ATP} channels by 66%, compared with an IC_{50} of $24\mu M$ against the K_{ACh} current. Since $2\mu M$ -the lowest concentration tested on the K_{ACh} current - caused a block of only $\sim 7\%$ of the adenosine induced current it seems unlikely that there is any significant contribution of K_{ATP} channels to the adenosine response in neonatal rat atrial myocytes.

6.5: DOES THE SUR ASSOCIATE WITH AND MODIFY INWARDLY RECTIFYING K^+ CHANNELS OTHER THAN K_{ATP} ?

The effects of glibenclamide are thought to occur through its interaction with the sulphonylurea receptor. Thus if the SUR was to combine with other inward rectifier channel proteins this might produce a mechanism whereby

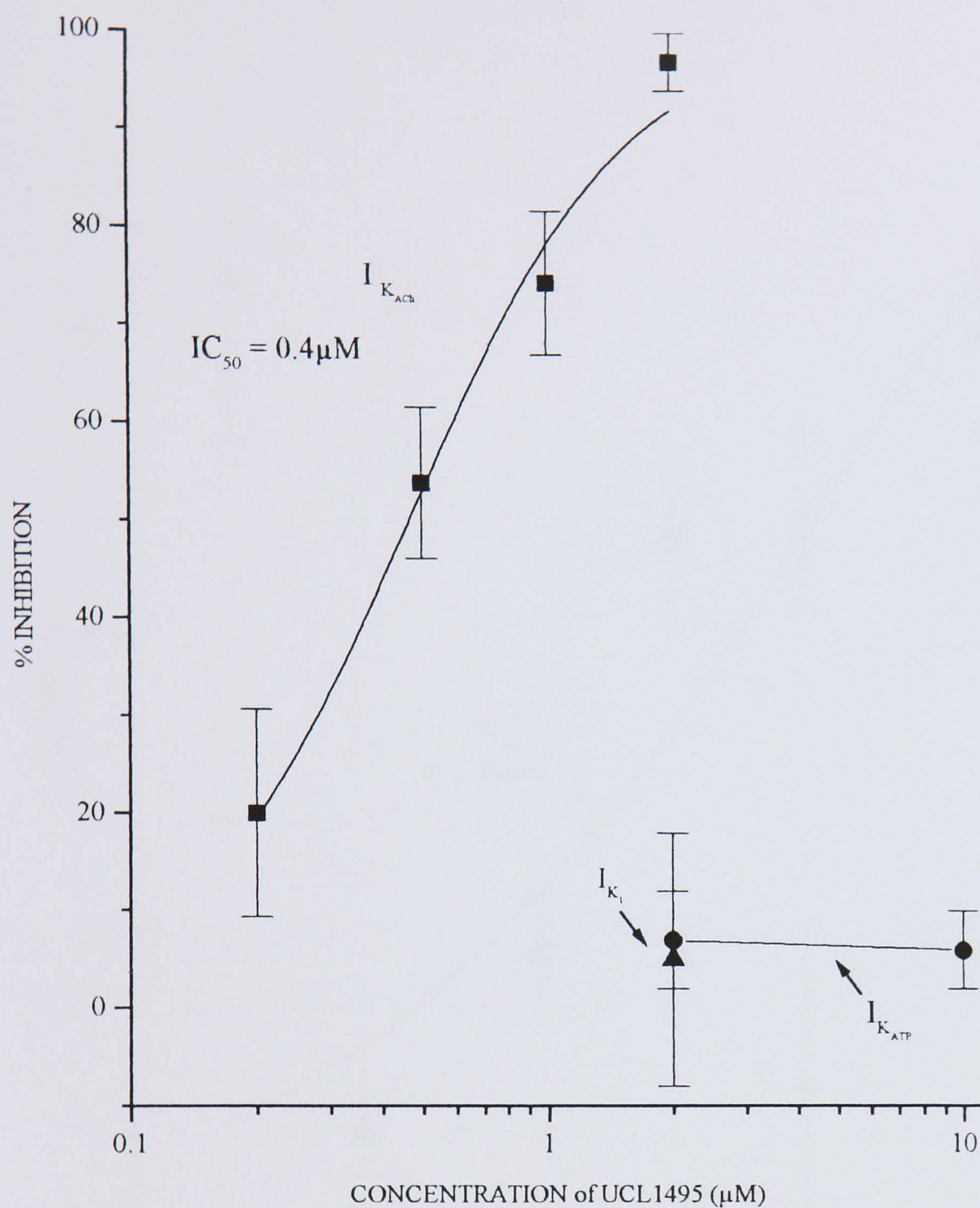


Figure 6.2: The effect of UCL1495 on the $I_{K_{ACh}}$, $I_{K_{ATP}}$ and I_{K_1} currents in neonatal rat myocytes.

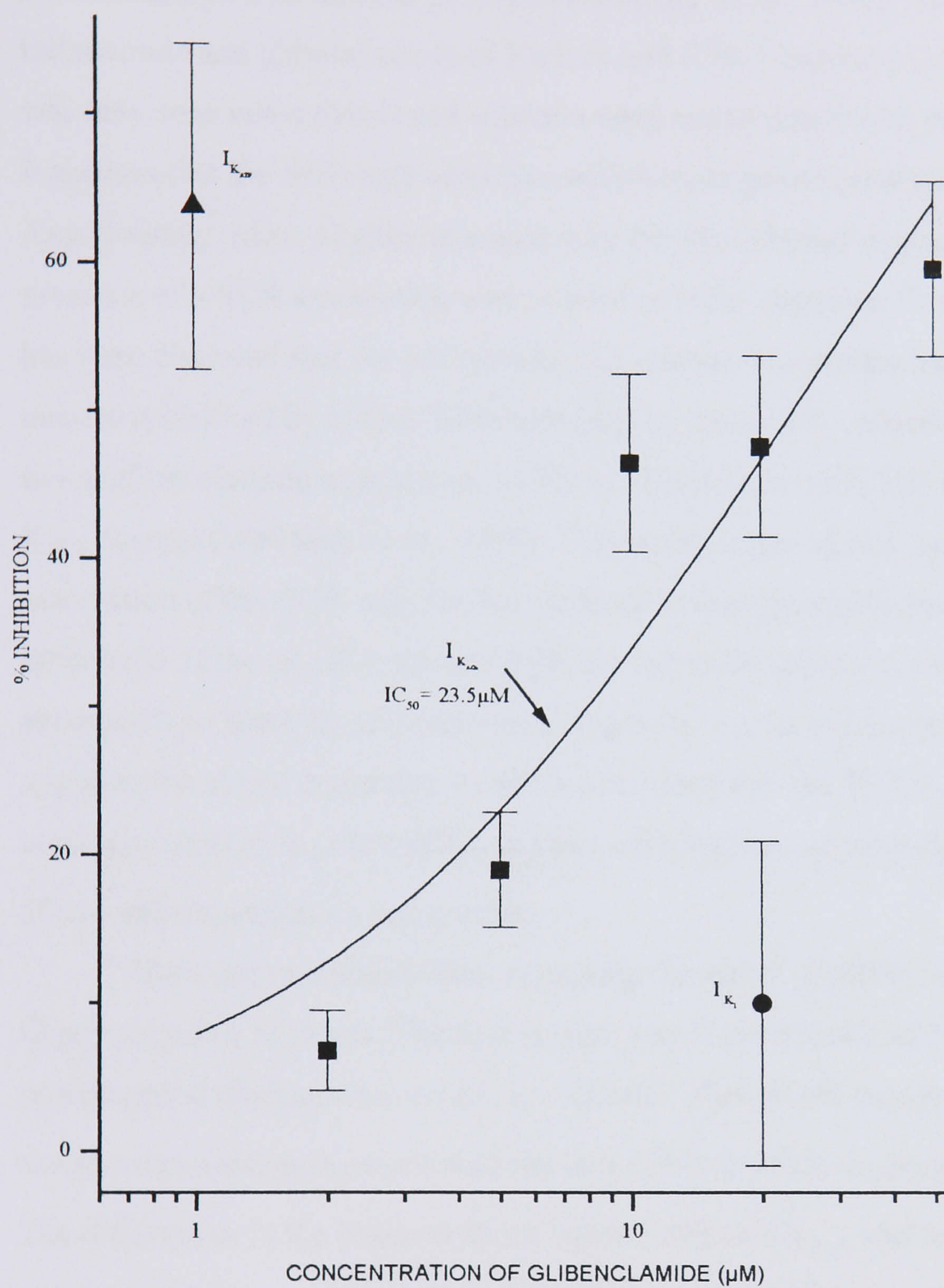


Figure 6.3: The effect of glibenclamide on the $I_{K_{Ac}}$, $I_{K_{ATP}}$ and I_{K_1} in neonatal rat myocytes.

glibenclamide could modify their activation. It has been suggested that the SUR associates with an endogenous Kir in HEK293 cells as the current was inhibited by tolbutamide with an IC_{50} of $5.3\mu M$ (Ämmälä *et al.*, 1996). Also block by tolbutamide and glibenclamide of Kir1.1a and Kir6.1 expressed in these cells, was only seen when the cloned subunits were coexpressed with the SUR. Thus it appears that the SUR may associate with Kirs in general and not just Kir6.2. Any blocking effect of glibenclamide may therefore be indicative of the presence of a SUR associating with inward rectifier channels. Contrastingly, it has been observed that the endogenous K_{IR} current in coronary vascular smooth muscle is blocked by $100\mu M$ glibenclamide by only 16% - whereas in the same tissue glibenclamide concentrations below $10\mu M$ were sufficient to block the K_{ATP} current (Wellman *et al.*, 1996). This would argue against any general association of the SUR with the K_{IR} channel. It seems possible that the SUR in atrial cells of the rat can associate with the G-protein gated channel making it susceptible to block by glibenclamide. From the results in Chapter Four, and applying the above argument, it can be concluded that the SUR does not associate with the I_{K_1} channel in rat atrial cells because of the lack of effect of $50\mu M$ glibenclamide on this current.

There are two possibilities regarding the effect of glibenclamide on the G-protein gated channels. The first is that there is association of SUR with the G protein-gated channels but not the I_{K_1} channel. This would explain the block of the adenosine induced current and the lack of block of the I_{K_1} by glibenclamide. The differences in the concentrations needed to block $I_{K_{ACh}}$ and $I_{K_{ATP}}$ may be due to the binding to the channels of different SURs which each have their own affinity for glibenclamide.

The interpretation that the block of the adenosine-induced current by glibenclamide is due to adenosine activation of K_{ATP} is dependent upon the supposition that glibenclamide is specific for K_{ATP} channels. This may be the second possibility : it may be, in fact, that glibenclamide is not as selective as it was first thought. It is possible that glibenclamide blocks the G-protein-gated channel directly. Phentolamine, an α -adrenoceptor antagonist, has been observed to block the wild type Kir6.2/SUR1 and the Kir6.2 independent of

SUR1 currents expressed in *Xenopus oocytes* with similar potencies of $\sim 1\mu\text{M}$ (Proks and Ashcroft, 1997). The Hill coefficients were ~ 1 suggesting a 1:1 interaction between drug molecule and binding protein. It was concluded that phentolamine binds with the Kir6.2 protein to cause inhibition. A blocking action of glibenclamide may, therefore, not be indicative of either K_{ATP} or a SUR, but may be due, instead, to the sulphonylurea blocking the K^+ channel directly.

6.6: THE EFFECT OF BARIUM:

Ba^{2+} blocks all three currents and is most potent on the I_{K_1} current with an IC_{50} of $17\mu\text{M}$ at -120mV . The effects of Ba^{2+} are summarised in Figure 6.4. The effect of voltage was difficult to determine due to the lack of any significant current at the holding potential of -50mV . An increased current would have been obtained by using a higher $[\text{K}^+]_0$ or by holding at 0mV and investigating the currents at -50 and -10mV . Ba^{2+} blocks the adenosine-induced current with an IC_{50} of $35\mu\text{M}$ at -50mV and $3.8\mu\text{M}$ at -120mV . This is in keeping with other studies which have also demonstrated that the block by Ba^{2+} is dependent on voltage and on the concentration of external K^+ , indicating that Ba^{2+} blocks the open channel (Standen *et al.*, 1978, Zang *et al.*, 1995, Carmeliet and Mubagwa). The dependence on external K^+ may also indicate that both Ba^{2+} and K^+ share a common binding site within the channel pore. Ba^{2+} blocked the levcromakalim-induced current at -50mV with an IC_{50} of $165\mu\text{M}$ which is close to reported values such as $180\mu\text{M}$ at -62mV in pancreatic β -cells (Takano and Ashcroft, 1996). Single channel studies in frog skeletal muscle show that Ba^{2+} decreases the mean channel open time suggesting a block of the open channels (Quayle *et al.*, 1988).

The blocking action of Ba^{2+} is known to be dependent on the presence of specific amino acid residues within the channel peptides. For example expressed Kir2.3/HRK1 channels which are strongly rectifying are blocked by external Cs^+ , with an IC_{50} of $50\mu\text{M}$, and by Ba^{2+} with an IC_{50} of $60\mu\text{M}$. Kir1.1a/ROMK1

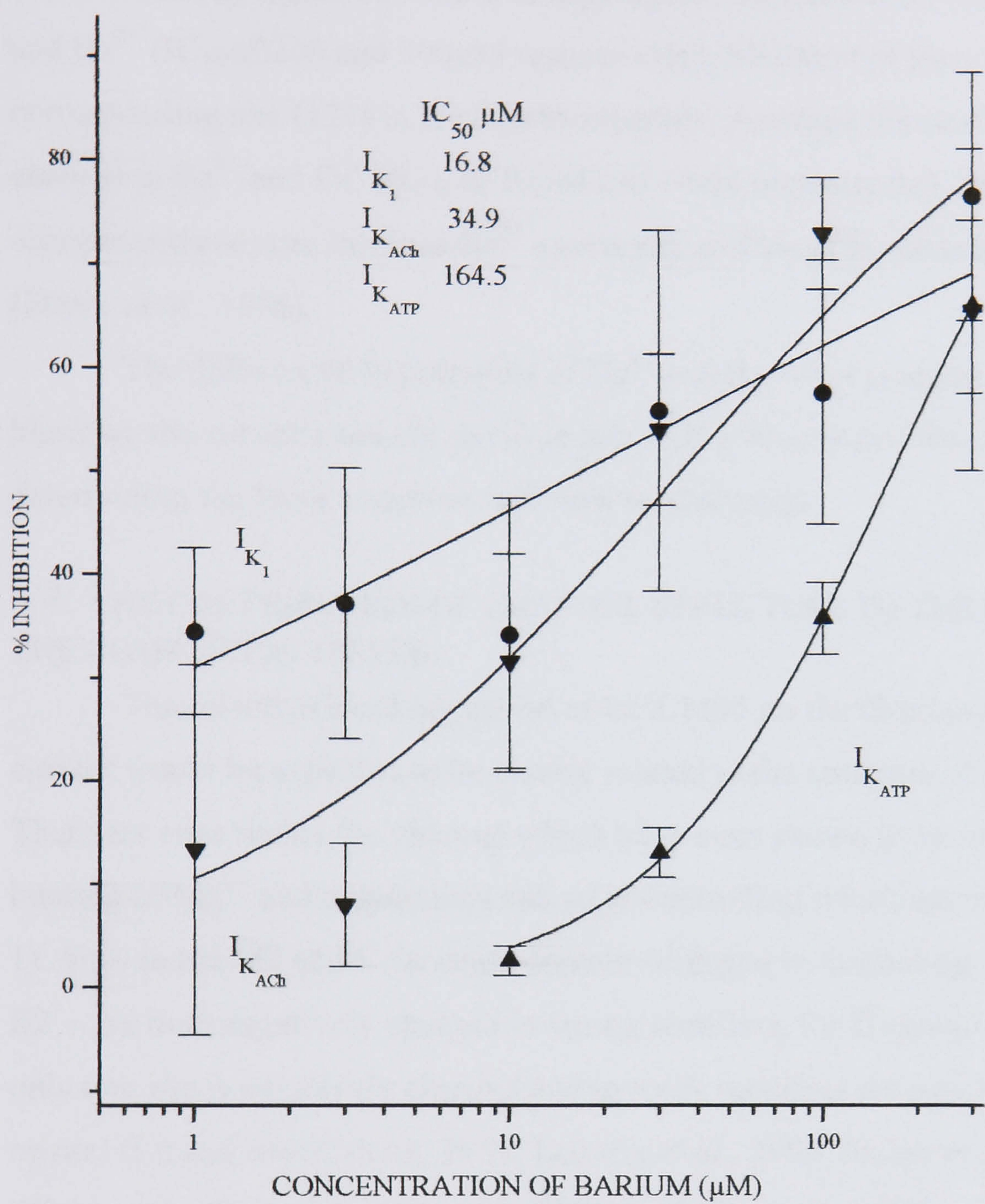


Figure 6.4: The effect of Barium on I_{K_{ACh}}, I_{K_{ATP}} and I_{K1} in neonatal rat myocytes.

channels which are weak rectifiers are blocked by Ba^{2+} with an IC_{50} of $50\mu\text{M}$, and are almost insensitive to Cs^+ with an IC_{50} of $\sim 3\text{mM}$. Mutation of aspartate (164) in the M2 region of Kir2.3 to asparagine reduced the sensitivity to Cs^+ and Ba^{2+} (IC_{50} of 200 and $300\mu\text{M}$ respectively). Mutation of asparagine at the corresponding site (171) in Kir1.1a to aspartate increased the sensitivity of the channel to Ba^{2+} and Cs^+ (IC_{50} of $10\mu\text{M}$ and 1mM respectively). Thus negative charges at these sites increase Ba^{2+} sensitivity and block by external cations (Henry *et al.*, 1996).

The differences in potencies of Ba^{2+} and the other compounds in blocking the currents and the possible role of the structure of the channels in determining the block observed will now be discussed.

6.7: THE CONTRIBUTION OF CHANNEL STRUCTURE TO THE EFFECTS OF THE COMPOUNDS TESTED.

The selective blocking action of UCL1495 on the G-protein-gated current would be expected to be closely related to the structure of the channel. There are sites within the channel which have been shown to be important in the binding of Mg^{2+} and polyamines and so in controlling rectification (see Chapter 1). Sites in the M2 and C-terminal domain -referred to as binding sites R1 and R2 - are both negatively charged in strong rectifiers, for G protein channels only one site is negatively charged and in weak rectifiers the amino acids are neutral (Lu and MacKinnon, 1994; Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Wible *et al.*, 1994; Stanfield *et al.*, 1994; Yamada *et al.*, 1995). It is highly likely that there are sites in the channel which determine the block by the novel compounds tested in this study. Identifying these sites requires one to know whether the drugs block in ionised or unionised forms and whether they block from the inside or the outside of the membrane, or from within it. This information has still to be obtained. Using our present knowledge of the structural determinants of rectification it can be speculated that if the drugs block from the inside then the channels with negative residues at both R1 and R2, i.e. I_{K_1} , will be most potently blocked by the compounds and the channels with neutral residues at these sites i.e. K_{ATP} should be the least blocked in accordance with this theory.

UCL1495 is rather potent against the $I_{K_{ACh}}$ and at the concentrations tested had no blocking effect on the other two currents. UCL1880 is also the most potent of the compounds tested in blocking the G protein gated current and is ~25 times more active than on the $I_{K_{ATP}}$ current. There is also a small block of the I_{K_1} current at higher concentrations. The results from the Ba^{2+} experiments showed that Ba^{2+} was most potent on the I_{K_1} , then $I_{K_{ACh}}$ and least potent on $I_{K_{ATP}}$. These findings with Ba^{2+} are in keeping with what is known about the residues present in the R1 and R2 positions. UCL1880 blocks with a quite different order of potency and so it may be concluded that the residues present at R1 and R2 are not important for its blocking action. UCL1495 is selective for the adenosine-induced current and has no effect on the other two. Thus it is possible that these residues are again not important.

The Hill slopes can give information (albeit indirectly) regarding the number of drug molecules binding to the channel. UCL1880 has a Hill slope of ~1 which at face value indicates a 1:1 interaction between the drug molecule and the blocking site. The same applies to glibenclamide which has a Hill slope of 0.8. For UCL1495 the Hill slope is 1.7, significantly greater than 1. This may mean either that more than 1 molecule binds to the site of interaction or that positive co-operativity occurs.

The Hill slope for Ba^{2+} block of $I_{K_{ACh}}$ is 0.6 at -50mV and 0.5 at -120mV. For I_{K_1} the slope is only 0.2 at -120mV. For $I_{K_{ATP}}$ the slope is 1.2. Thus for $I_{K_{ATP}}$ and perhaps $I_{K_{ACh}}$ there may be a 1:1 interaction of Ba^{2+} with its binding site but for I_{K_1} the data is inconsistent with so simple a scheme. It is unlikely that there is 'negative co-operativity'. The data for I_{K_1} in Figure 6.4 which summarises the effect of Ba^{2+} on the three inward rectifiers may be fitted with 2 components though the precision of the data is too low to allow this to be done with any certainty. It is unlikely that Ba^{2+} has 2 binding sites with different affinities as it has been well established that Ba^{2+} has a 1:1 interaction at a site within the channel pore (Zang *et al.*, 1995, Carmeliet and Mubagwa, 1996). It seems more likely that Ba^{2+} is blocking 2 populations of K^+ channels. As mentioned previously in Chapter 4 there is activation of I_f as well as I_{K_1} on

stepping to hyperpolarising potentials. Whilst it is generally considered that these channels are present in the cells of the sino-atrial node where they are involved in the pacemaker activity (DiFrancesco, 1993) a hyperpolarisation activated current has also been described in atrial tissues (Thuringer *et al.*, 1992). Another possibility is that some sino-atrial node cells may have been present in the culture. Ba^{2+} will block the two different channels with different affinities.

The results of this study have revealed that the IC_{50} values for the block of the channels by Ba^{2+} are in keeping with the amino acids present at the important positions in the M2 and C-terminal domain. The Hill slopes for block of $I_{K_{ACh}}$ and $I_{K_{ATP}}$ suggest a 1:1 interaction of the Ba^{2+} ion and its binding site within the channel. As described there is a voltage-dependent effect for Ba^{2+} block of $I_{K_{ACh}}$ which suggests that the ion blocks the channel within the voltage field, some distance from the external side of the membrane. The low Hill slope for the block by Ba^{2+} of I_{K_1} may be due to the block of another component, possibly I_f , in addition to block of I_{K_1} .

Comparing the blocks by the other compounds with that by Ba^{2+} suggests, in all likelihood, that the amino acid residues which are important in Ba^{2+} block in the three channels are not as important for the block caused by UCL1880, UCL1495 and glibenclamide. The increased potency of UCL1880 and UCL1495 on the steady state current, and the use-dependent effect data suggest a block of the channels.

6.8: FUTURE WORK.

Single channel recordings should prove useful in establishing whether or not the compounds UCL1880 and UCL1495 block the channel directly, as the results suggest. The novel compound UCL1880 has proven to be very potent against the G-protein-gated current and exhibits considerable selectivity. Investigating its effect on other K^+ -currents – indeed, as many other currents as possible- would allow a profile of its selectivity to be built up. It is possible that it will prove a useful tool in investigating cardiac K^+ currents if it is indeed as

selective as appears from my work. Single channel recordings would also aid in determining if the activation of the G-protein-gated muscarinic K^+ channel includes activation of $I_{K_{ATP}}$. It is possible that the block of a current under investigation by glibenclamide does not necessarily indicate the presence of $I_{K_{ATP}}$. Mutational studies would be useful in identifying the residues within the structures of these 3 channels that determine the degree of inhibition observed. Finding how UCL1880 interacts with its binding site in the G-protein-gated channel – be it a positively or negatively charged residue – could aid the future development of compounds of greater potency and selectivity.

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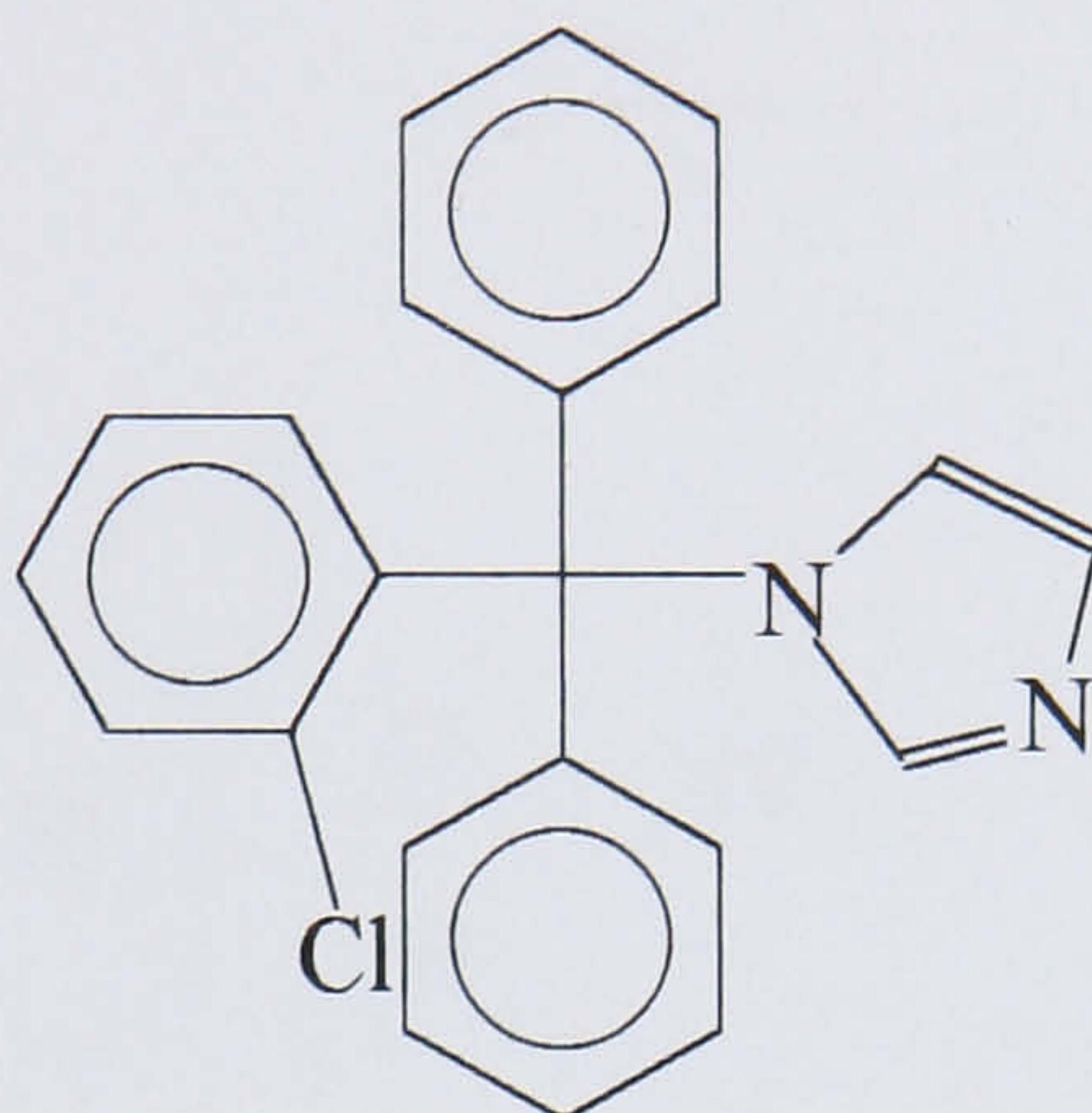
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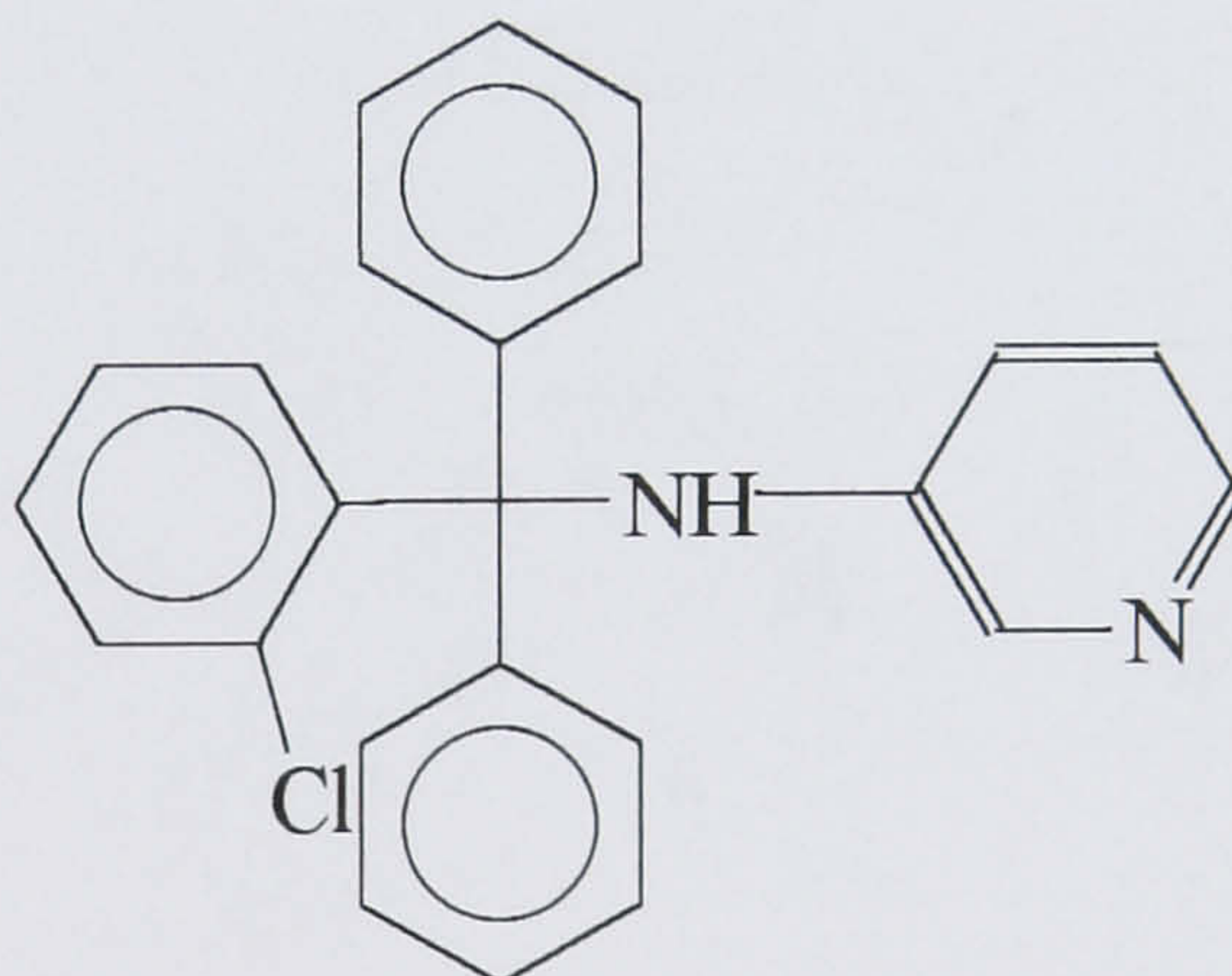
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The structures of clotrimazole and UCL1880.

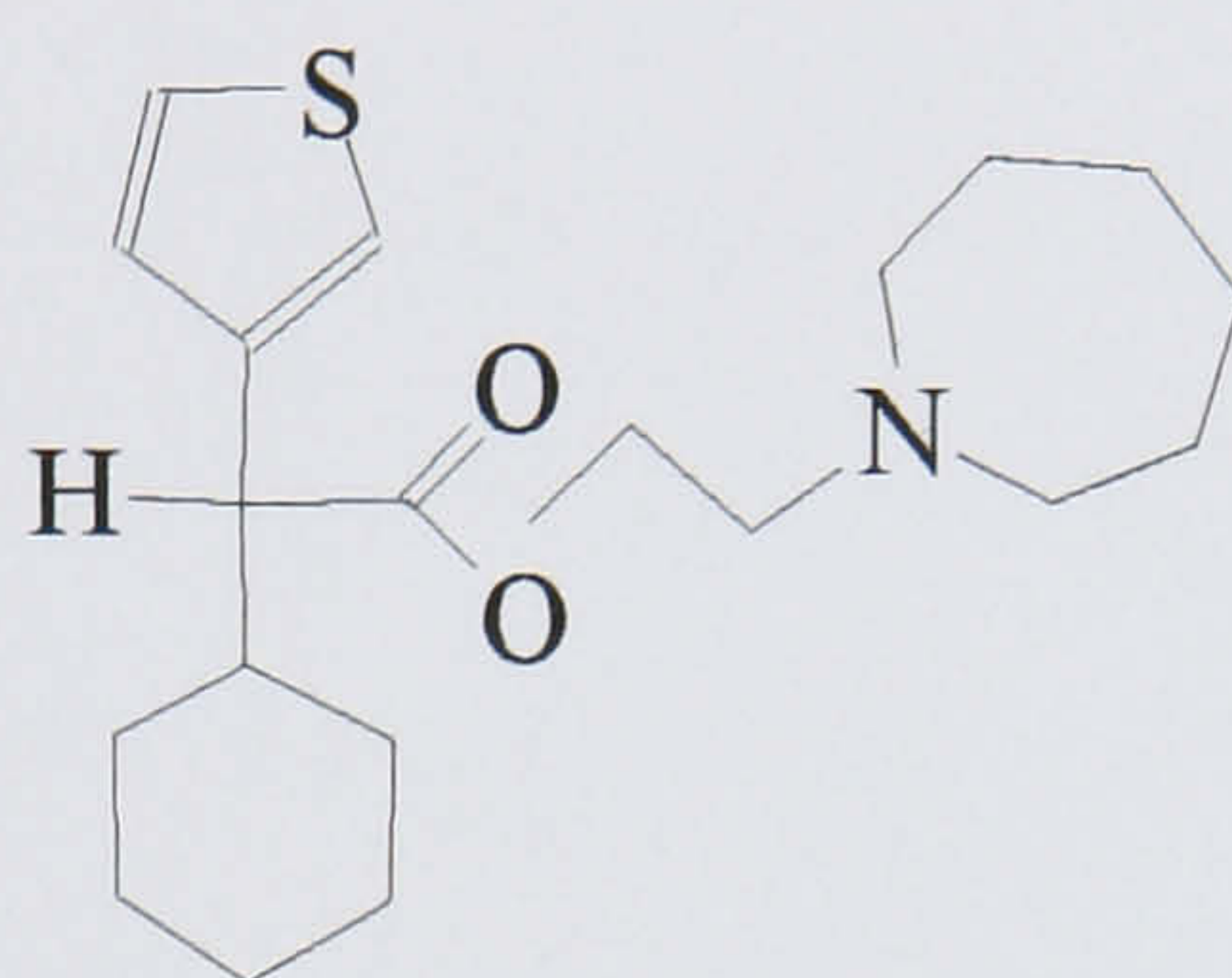


Clotrimazole

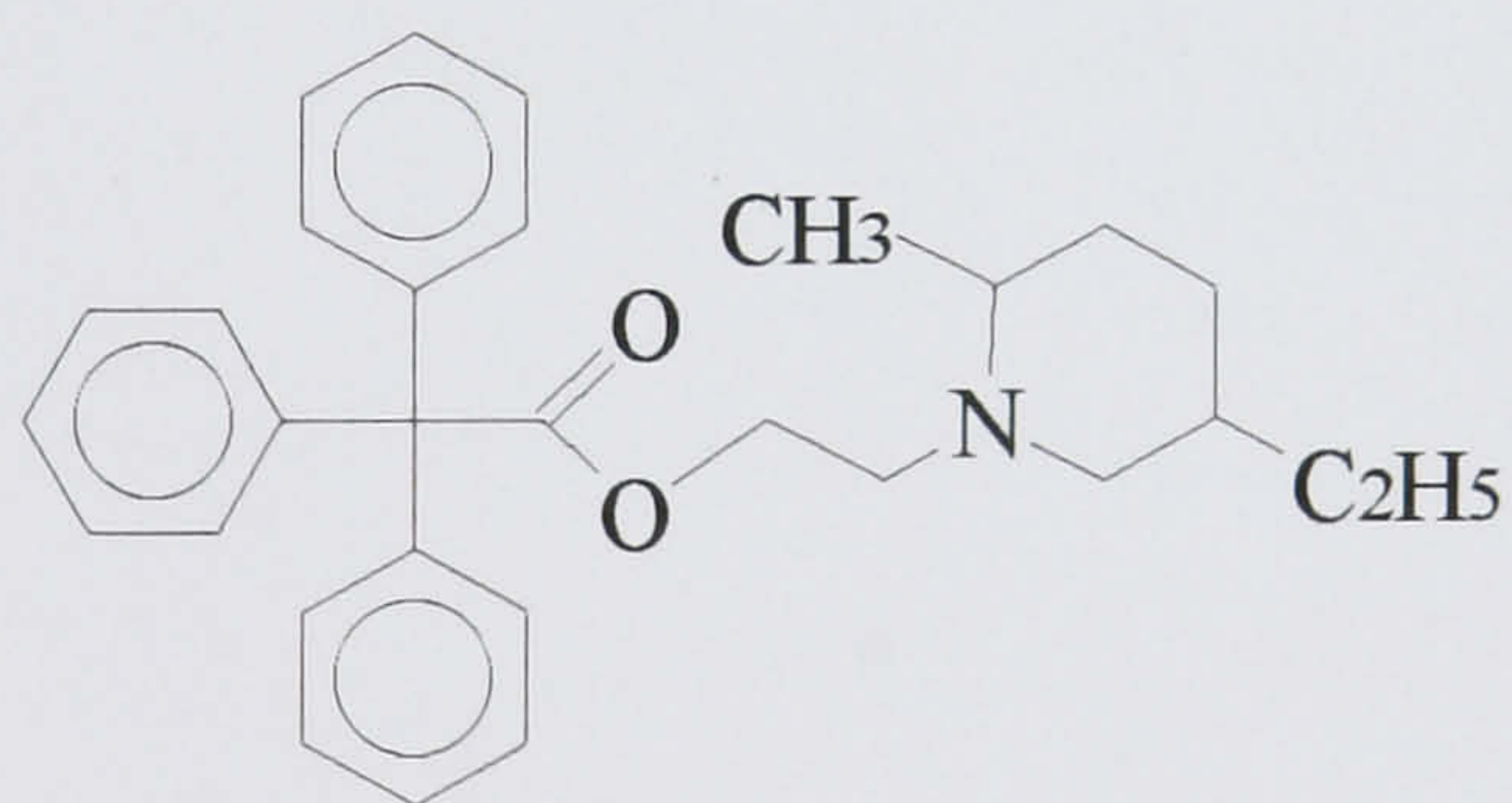


UCL1880

The structure of Cetiedil and UCL1495



Cetiedil



UCL1495